Multiparameter flow cytometric immunophenotyping of epithelial cells in effusions

Technical improvements and apoptosis measurement

Hiep Phuc Dong

Division of Pathology, Norwegian Radium Hospital,

Oslo University Hospital

National Resource Center for Women's Health, Rikshospitalet,

Oslo University Hospital

Faculty of Medicine,

University of Oslo

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1496

ISBN 978-82-8264-212-5

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Akademika publishing.
The thesis is produced by Akademika publishing merely in connection with the thesis defence. Kindly direct all inquiries regarding the thesis to the copyright holder or the unit which grants the doctorate.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This thesis was performed at the Division of Pathology in collaboration with the Department of Gynecologic Oncology, Division of Gynecology and Obstetrics, at the Norwegian Radium Hospital, Oslo University Hospital. I gratefully acknowledge the Inger and John Fredriksen Foundation for Ovarian Cancer Research, the Norwegian Cancer Society and the Norwegian Radium Hospital Research Foundation for financially supporting the studies in my thesis.

I wish to express my deepest gratitude to my main supervisors at the Division of Pathology, Norwegian Radium Hospital.

To Prof. Ben Davidson, for introducing me to the exciting field of effusion cancer research, sharing your knowledge and enthusiasm, your constructive and valuable guidance for each paper and the thesis. I value your friendship, encouragement, support and help.

To Dr. Bjørn Risberg, for inspiring me to enter the interesting field of flow cytometry. Your valuable comments and discussions regarding the technical aspects of flow cytometry were very helpful. I appreciated your friendship, inspiration and encouragement.

I would like to thank Prof. Jahn M. Nesland, the former Head of the Department of Pathology, for supporting and encouraging me to perform this thesis.

To Prof. Claes G. Tropé, Head of Research at the Division of Gynecology and Obstetrics, for providing the financial support from the Inger and John Fredriksen Foundation for Ovarian Cancer Research, for his clinical contribution and enabling the use of patient material in this thesis.

Special thanks to Dr. Lilach Kleinberg and Mr. Arild Holth for your friendship and valuable collaboration. Warm appreciation to the work of my co-authors Prof. Aasmund Berner, Prof. Vivi Ann Flørenes, Ms. Marit Gunhild Ruud, Ms. Elisabeth Emilsen, Ms. Anne Katrine Ree Rosnes, Ms. Annika Jøntvedt Bock, Dr. Ilvars Silins and Dr. Mari Bunkholt Elstrand. Thank you for your contribution.

I would like to thank all the friends and colleagues at the Department of Pathology, Norwegian Radium Hospital. Special thanks to Dr. Hari Prasad Dhakal, the staff at Section of Cytology, the staff at the Micrometastasis Laboratory and the staff at the Flow Cytometric Laboratory, for their friendly encouragement and support, and Dr. Anne Tierens for sharing her flow cytometric expertise with me.

Sincere thanks to my “Danish family”- Elise, Lillian, Børge, Kaj, Grethe and Annika for their love, support and encouragement and for providing me such a wonderful environment during my childhood in Denmark.

I am grateful for the support, encouragement and help of all the in-laws and friends.

Finally, I would like to express my deepest gratitude to my parents and brothers and their family for their enduring love, encouragement and support. To my beloved wife, Hong, for your love, patience, understanding and believing in me, and to our beloved children Helen and Henrik for bringing happiness and joy into our family.

Oslo, August 2012

Hiep Phuc Dong

ABBREVIATIONS

AC	Metastatic adenocarcinoma
AIF	Apoptosis-inducing factor
AKT	v-Akt murine thymoma viral oncogene homolog
APAF-1	Apoptotic protease-activating factor-1
APC	Allophycocyanin
Apo2.7	Mitochondrial membrane protein 7A6 antigen
ASP-XXX	Aspartic acid
ATCC	American Type Culture Collection
B72.3	Murine IgG monoclonal antibody to recognize tumor associated glycoprotein 72
BAD	Bcl-2-associated death promoter homolog
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2 associated X-protein
BCL-2	B-cell chronic lymphocytic leukemia/lymphoma 2
BCL-W	B-cell chronic lymphocytic leukemia/lymphoma-w protein
BCL-XS	Bcl-2-like 1 protein (short form)
BCL-XL	Bcl-2-like 1 protein (long form)
BD	Becton-Dickinson
BER-EP4	Epithelial cell adhesion/activating molecule antibody
BFL-1	Bcl-2-related protein A1
BG-8	Blood Group Related antigen-8
BID	BH3 interacting-domain death agonist
BIK	Bcl-2-interacting killer
BIM	Bcl-2-like protein 11
BMF	Bcl-2-modifying factor
BOK	Bcl-2-related ovarian killer protein
BP	Bandpass filter
bp	base pairs
BRCA1/2	Breast cancer gene 1/2
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CA-125	Cancer antigen-125
CA19-9	Carbohydrate antigen 19-9

CARD	Caspase activation and recruitment domain
CCNE1	Cyclin E1
CD45	Cluster of differentiation antigen 45
CD138	Cluster of differentiation antigen 138
CEA	Carcinoembryonic antigen
cDNA	Complementary deoxyribonucleic acid
c-FLIP$_{S/L}$	Cellular FLICE-inhibitory protein (short/long isoform)
CGH	Comparative genomic hybridization
CK5/6	Cytokeratin 5/6
CT	Computed tomography
CTNNB1	Catenin (cadherin-associated protein), beta 1
CYC	Cytochrome c
DAB	3,3´-diaminobenzidine
DD	Death domain
DED	Death effector domain
DNA	Deoxyribonucleic acid
DCR	Decoy receptor
$DiOC_6(3)$	3,3-dihexiloxa-dicarbocyanine
DISC	Death-inducing signaling complex
DMSO	Dimethylsulfoxide (Me_2SO)
DR	Death receptor
DSB	Double-strand breaks
dUTP	Deoxyuridine triphosphate
EDAR	Ectodysplasin A receptor
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
EndoG	Endonuclease G
EORTC 55971	European Organization for Research and Treatment of Cancer 55971
EpCAM	Epithelial cell adhesion/activating molecule
ER	Endoplasmic reticulum
ERBB2	v-Erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated kinase
Ets	E-twenty six
FADD	Fas-associated death domain

FAS/CD95	Tumor receptor superfamily, member 6/Cluster of differentiation antigen 95
FASL	Fas ligand
FBS	Fetal bovine serum
FCM	Flow cytometry
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FL	Fluorescent channel
FLICA	Fluorochrome-labeled inhibitors of caspases
FRA	Folate receptor alpha
FSC	Forward scatter channel
GEP	Granulin-epithelin precursor
GOG218	Gynecologic Oncology Group 218
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
HE4	Human Epididymis protein 4
HER-2	Human epidermal growth factor receptor-2
HGS-ETR-1	Mapatumumab
HGS-ETR-2	Lexatumumab
HIF	Hypoxia inducible factor
HMBE-1	Monoclonal anti-mesothelial cell, clone HMBE-1
HRK	Activator of apoptosis Harakiri
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
IC_{50}	Half maximal inhibitory concentration
ICC	Immunocytochemistry
ICE	Interleukin-1βeta converting enzyme
ICON7	International Collaborative Ovarian Neoplasm-7
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-8	Interleukin-8
IP	Intraperitoneal

ITCH	A HECT-type E3 ligase
IV	Intravenous
JC-1	3,3-tetraethylbenzimiazolcarbocyanie iodide
kDa	Kilodalton
KRAS	Kirsten rat sarcoma 2 viral oncogene homolog
LDH	Lactate dehydrogenase
Leu-M1(CD15)	Cluster of differentiation antigen 15
LP	Longpass Filter
MAPK	Mitogen-activated protein kinases
MCL-1	Myeloid cell leukemia sequence 1
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MM	Malignant mesothelioma
MMP	Matrix metalloproteinase
MOC-31	Epithelial-related antigen clone MOC-31
mTOR	Mammalian Target of Rapamycin
MUC4	Mucin 4, cell surface associated
MYC	v-Myc avian mylocytomatosis viral oncogene homolog
NaCl	Natrium chloride
NACT	Neoadjuvant chemotherapy
NF-κB	Nuclear factor κB
NGFR	Nerve growth factor receptor
OC	Ovarian carcinoma
Omi/HtrA2	HtrA serine peptidase 2
OPG	Osteoprotegrin
OS	Overall survival
p53wt	p53 wild type
$p75^{NGFR}$	Nerve growth factor receptor
PAP	Papanicolaou stain
PARP	Poly (ADP-ribose) polymerase
PBL	Peripheral blood leukocytes
PCR	Polymerase chain reaction
PerCP	Peridinin chlorophyll protein

PFS	Progression-free survival
PI	Propidium iodide
PI3K	Phosphatidylinositol-3 kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic alpha
PKB	Protein kinase family
PMT	Photomultiplier tube
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog protein encoded by the *PTEN* gene
PUMA	p53 upregulated modulator of apoptosis
RAS	Rat sarcoma superfamily
RIP	Receptor-interacting protein
RM	Reactive mesothelium
RNA	Ribonucleic acid
RPE	R-phycoerythrin
RPM	Rounds-per-minute
RPMI	Roswell Park memorial Institute
Rsf-1	Remodeling and spacing factor 1
SDS	Sodium dodecyl sulfate
sFAS	Soluble Fas
siRNA	Small interfering ribonucleic acid
Smac/DIABLO	Second mitochrondria-derived activator of caspase/direct IAP-binding protein with low pI
S/N	Signal-to-noise
SSC	Side-scatter channel
TBST	Tris buffered saline-tween
TEM	Transmission electron microscopy
TGF-β1	Transforming growth factor-β1
TKI	Tyrosine kinase inhibitor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TP53	Tumor protein 53
TRA-8	Agonist monoclonal antibody
TRADD	TNFR-associated death domain protein

TRAF 1/2	TNFR-associated factor 1/2
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRM-1	TRAIL-R1 monoclonal antibody
TTF-1	Thyroid transcription factor-1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
TVS	Transvaginal ultrasonography
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
v-FLIP	Viral FLICE-inhibitory protein
WB	Western blotting
WT1	Wilms tumor-1
XIAP	X-linked inhibitor of apoptosis
7-AAD	7-amino-actinomycin-D

LIST OF PUBLICATIONS

Paper I:

Dong HP, Holth A, Berner A, Davidson B, Risberg B. **Flow cytometric immunphenotyping of epithelial cancer cells in effusions--technical considerations and pitfalls.** Cytometry B Clin Cytom 2007;72:332-343.

Paper II:

Dong HP, Kleinberg L, Silins I, Flørenes VA, Tropé CG, Risberg B, Nesland JM, Davidson B. **Death receptor expression is associated with poor response to chemotherapy and shorter survival in metastatic ovarian carcinoma.** Cancer 2008;112:84-93.

Paper III:

Dong HP, Kleinberg L, Davidson B, Risberg B. **Methods for simultaneous measurement of apoptosis and cell surface phenotype of epithelial cells in effusions by flow cytometry.** Nat Protoc 2008;3:955-964.

Paper IV:

Dong HP, Holth A, Kleinberg L, Ruud MG, Elstrand MB, Tropé CG, Davidson B, Risberg B. **Evaluation of cell surface expression of phosphatidylserine in ovarian carcinoma effusions using the annexin-V/7-AAD assay: clinical relevance and comparison with other apoptosis parameters.** Am J Clin Pathol 2009;132:756-762.

Paper V:

Dong HP, Holth A, Ruud MG, Emilsen E, Risberg B, Davidson B. **Measurement of apoptosis in cytological specimens by flow cytometry: comparison of Annexin V, caspase cleavage and dUTP incorporation assays.** Cytopathology 2011;22:365-372.

Paper VI:

Dong HP, Ree Rosnes AK, Bock AJ, Holth A, Flørenes VA, Trope' CG, Risberg B, Davidson B. **Flow cytometric measurement of cellular FLICE-inhibitory protein (c-FLIP) in ovarian carcinoma effusions.** Cytopathology 2011;22:373-382.

1. INTRODUCTION

1.1. Flow cytometry

Flow cytometry (FCM) is an indispensable tool for diagnosis, classification, staging and monitoring of hematological malignancies (1,2). Improvements in FCM instrumentation and the availability of an expanded range of antibodies and fluorochromes render it an optimal tool for the study of other difficult biological and diagnostic issues. Recent refinements and improvements in FCM technology have provided new investigative approaches and improved accuracy in the diagnosis of hematological neoplasms (1,2). However, the application of this technique to the diagnosis and clinical research of non-hematological cancers, e.g., in effusion cytology, still requires improvement in way of optimization and calibration, due to the different characteristics of epithelial cells compared to lymphoid cells. Multiparameter FCM immunophenotyping is a suitable method in terms of rapidity, sensitivity, reproducibility and reliability for measurement of cellular antigens, including those located in the cytoplasm, nucleus, and cell surface. This technology allows simultaneous measurement and quantification of various cellular attributes, contributing to an accurate definition of antigen profiles on diverse cell populations.

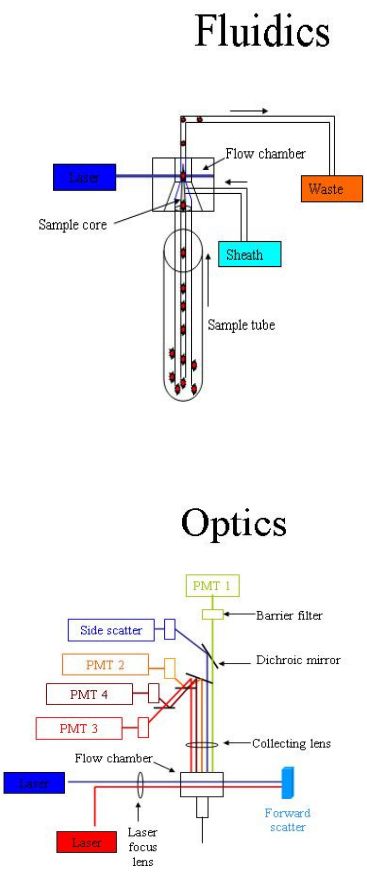
1.1.1 The principles of FCM

FCM is a technology for measuring various properties of cells, cell organelles, and other objects suspended in liquid and flowing at rates of several thousand per second through a flow chamber in which a technique called hydrodynamic focusing organizes the cells into a single stream. This stream then passes through the point of interrogation, in which the cells can be exposed to a number of lasers with different wavelengths (3-11).

The applications of FCM are diverse and can be used to investigate whole cells and some of the cellular components, such as DNA, RNA, nuclear antigens, enzymes, surface antigens,

etc. Theoretically, any constituent of a cell to which a fluorescent dye can bind is measurable. The important feature is that measurements are made on single cells, individually.

A flow cytometer is composed of three major subsystems, which cooperate to simultaneously measure multiple physical characteristics of single cells/particles, as shown diagrammatically in **Figure 1**.



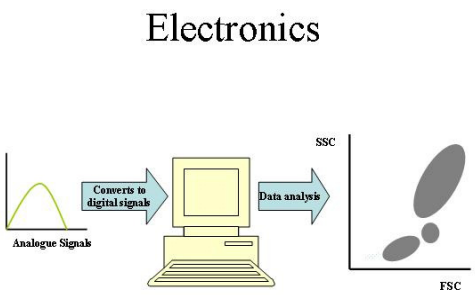


Figure 1. Diagrammatic representation of the three major subsystems in a flow cytometer.

1.1.2 The fluidics subsystem

The important task of the fluidics subsystem is to bring and center cells/particles in the illuminating beam. The flow chamber is the main component of the fluidics subsystem where the cells in suspension are slowly injected (inlet stream) into a faster flowing system of fluid (sheath fluid), providing a "laminar sheath" that surrounds the cells or particles (**Figure 2**). When the circumstances are right, sample fluid (the inlet stream) is confined to the central core of the laminar flow entering the interrogation point. This process is called hydrodynamic focusing. It stabilizes and narrows the sample stream containing cells through the interrogation point, where the cells intersect with excitation sources (3-9,11). The sample and sheath fluid streams may be driven by air pressure, by vacuum or by pumps. The majority of commercial flow cytometers use a closed flow chamber and air pressure (7,9,10). The overall velocity of the cell suspension through the chamber is determined by the pressure setting use to drive the sheath fluid. If the flow rate of the sheath fluid increases without a change in the core flow rate, the core diameter becomes smaller and the cells move faster. Conversely, if the sheath flow rate decreases without a change in the core flow rate, the core diameter becomes larger and the cells move slower (4,7,11). The flow rate of the cells passing the illumination

beam depends on the velocity of the sheath fluid stream, the diameter of the sample core, and the concentration of cells in the suspension. In most commercial flow cytometers the sheath flow rate can be adjusted. At a given laser beam size, the lower the flow rate, the more times each cell will be excited and the more light will be collected leading to increase the sensitivity. However, extended dwell time in the laser beam may result in significant photobleaching of many fluorochromes (4,7,11).

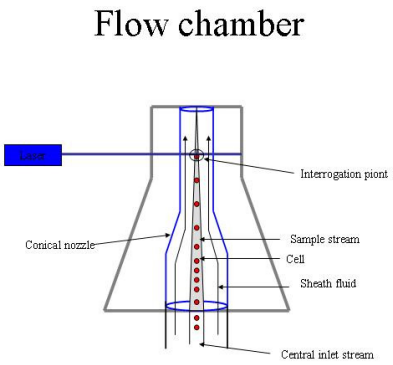


Figure 2. The Flow Chamber in which the cells in suspension are slowly injected (inlet stream) into a faster flowing system of fluid (sheath fluid), providing a "laminar sheath" surrounding the cells.

1.1.3 The optics subsystem

The optics subsystem is composed of excitation sources and components to collect light signals and direct them to the suitable detectors (**Figure 1**).

1.1.3.1 The excitation sources

Most current flow cytometers utilize lasers as light sources to illuminate a cell/particle that has been hydrodynamically focused by the fluidics system (**Figure 1**). The reason for using lasers is due to their ability to provide a narrow, intense beam of monochromatic light that can

be focused by a lens to a small point (interrogation point). Cells/particles in a sample stream can flow rapidly through this interrogation point receiving an adequate amount of illumination during their short time of exposure in a laser beam to generate scatter and fluorescence light of detectable intensity. The large majority of modern flow cytometers are equipped with more than one laser (up to four or more). The most commonly utilized laser is an argon ion laser configured to produce light at 488 nm wavelength. Due to the increasing demand for a wide array of fluorochomes, an increase in the number of lasers (helium-neon, violet, red diode, etc.) on flow cytometers is required (3-11).

1.1.3.2 Measurement of light signals

When a cell/particle intersects a laser beam, it scatters and emits light in all directions (360°). The optical subsystem is responsible for collecting light signals and routing them to the appropriate detectors. Two types of light scattering properties are measured in two directions. One is over a narrow angle in the forward direction, and second is at right angle to the laser beam by a photodiode and photomultiplier tube (PMT), respectively (3-7,11). These devices convert the light signal to an electrical signal that can be processed by the data processing and analysis unit.

The wavelength of the scattered light is the same as the incident light, and the laser light scattered by a cell/particle depends on its size and other properties. Scatter light characteristics yield valuable information about the sample and can be used to discriminate between different cellular subpopulations (e.g. lymphocytes, monocytes and granulocytes), cells from debris, viable cells from dead cells, and single cells from clusters of cells. The amount of light scattered over a narrow angle at approximately 0.5° to 10.0° in the forward direction is detected in the forward scatter channel (FSC). The intensity of forward scatter is proportional to the size, shape and optical homogeneity of cells, size being the predominant

parameter. The forward scatter gives strong signal that can be detected by a photodiode, which translates FSC light into electrical pulses. The electrical pulses are amplified and digitalized for storage in a computer.

The amount of light scattered to the side (perpendicular to the axis of the laser light, 90°) is detected in the side or right angle light scatter channel (SSC). The intensity of side scatter is proportional to the size, shape and optical homogeneity of cells, optical homogeneity being the predominant parameter (**Figure 3**). Side scatter signals are relatively weak and need to be amplified by a PMT, which converts SSC light to an output signal current and digitized for storage in a computer (3,4,11).

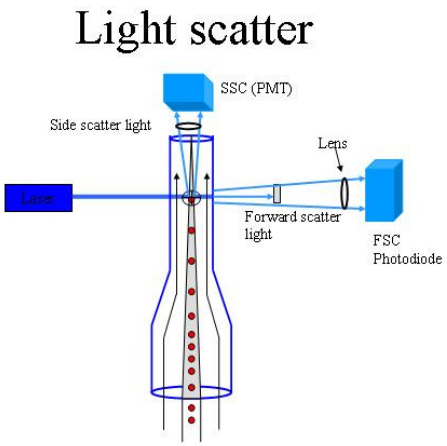


Figure 3. Schematic diagram of a flow cytometer with FSC and SSC detectors.

1.1.3.3 Fluorescent emission

Fluorescence (property of emitting electromagnetic radiation as a result of absorption of radiation [photons] by an atom, molecule or ion) (12) occurs when fluorochrome-labeled

cells/particles are excited by a laser beam of a certain wavelength (electrons can only remain in excited state for a few nanoseconds) returns to the unexcited (ground) state by emitting light of a longer wavelength (**Figure 4**) (3,12). The major advantage of using fluorochromes for FCM is their sensitivity. By combining high power lasers, efficient light-collecting optics and sensitive PMT a few thousand fluorochromes per cell can be specifically detected. Thus FCM is able to demonstrate or quantify low amounts of cellular macromolecules. In FCM, in addition to light scatter, one or more fluorescence signals are usually measured. The fluorescent light is collected at right angles to the laser beam. Most current laboratory flow cytometers are equipped with two or more lasers and are able to measure four or more fluorochromes simultaneously (13).

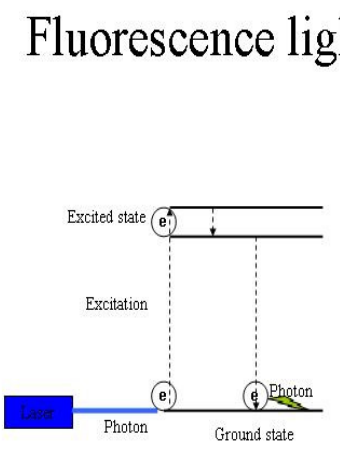


Figure 4. The absorption and emission of light during fluorescence.

Fluorescent molecule absorbs energy as photons, which increases the energy level of its electrons. However, when the molecule returns to ground state, it emits light (photons) of a longer wavelength than the excitation wavelength.

1.1.3.4 The optical subsystem

The optical system of a flow cytometer is responsible for collecting and quantifying the scatter light and emitted fluorescence. The different types of fluorescent emission - green, yellow, red and deep red - and the scattered light are collected and separated from one another by using networks of dichroic mirrors, beam splitters and optical filters, which can direct the emitted light with certain wavelength toward specific optical filters, before it can be detected by the PMTs and converted to electrical signals (**Figure 5**).

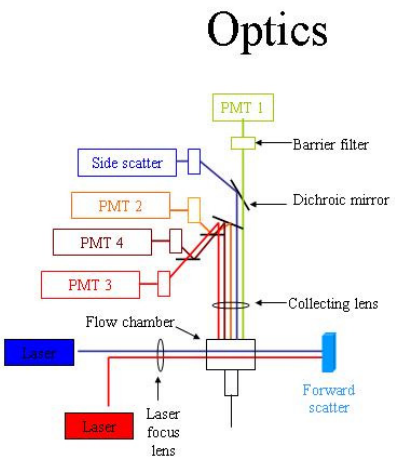


Figure 5. Optical layout.

Both side scatter light signal and fluorescence light signal are collected simultaneously through the same collector lens, The composite light signal has to be separated in its individual component by using dichroic mirrors, beam splitter and filters, before it can be detected by the PMT.

In flow cytometers with more than one laser, the laser beams are separated in a way that a cell flows through each laser beam within a few microseconds. Thus the signals are separated in time, making it easier to resolve them.

Each of the fluorescent channels is configured to measure a narrow range of wavelengths. PMTs are located at the end of each light path and are designated as FL1, FL2, FL3 and FL4 for detection of the green, yellow, red and deep red fluorescence, respectively. Each fluorochrome does not emit a single wavelength of light, but a broad spectrum of light. In single color analysis, fluorescence signal is detected in one channel corresponding to the wavelength emitted by the fluorochrome. In multicolor analysis with two or more different fluorochromes, problems with emission spectra overlaps occur and fluorescence signal from a single labeled cell/particle may be detected in two different channels, leading to false-positive results. To partly solve the emission spectra overlaps, band pass (transmitting a specific narrow band of wavelengths) are placed in front of each PMT in order to reduce spectral overlap. In principle, more lasers, detectors and different optical filters can be configured to make even more measurements on each cell, with the limitation being the number of dye combinations that can be used (3,4,6,11).

1.1.4 The electronic subsystem

The main function of the electronic subsystem is to convert scatter and fluorescence light signals to electronic signals, which are then digitized and further processed into numerical data. Data for each cell/particle will be stored in the computer for further analysis.

The main components of electronic circuitry of a flow cytometer are shown in **Figure 6**.

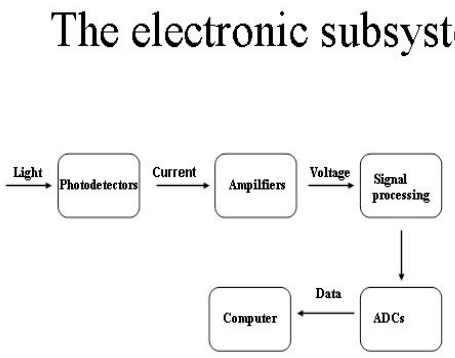


Figure 6. A schematic diagram of the electronic circuitry of a flow cytometer.

1.1.4.1 Signal processing

Photodetectors detect photons of light and produce electrical current. The electrical current is converted to a voltage by a preamplifier, which produces a voltage pulse that is proportional in size to the number of photons that originally reached the detector (4).

Threshold setting

Pulses are generated when cells/particles flow through a laser. In order to avoid collection of data on debris or noise in the system, a threshold (a minimum of pulse high for signals to be processed) can be applied on the output voltage so that only signals above a certain limit are processed. Signals below threshold are not processed and no data are acquired for those events (**Figure 7-A**). It is set by the operator, usually on the FSC parameter. **Figures 7- B, 7-C** and **7-D** show an example of the threshold setting after the signals are digitized and further processed into numerical data and transferred to the computer. If the threshold is set too low, small signal events such as debris and noise will be included (**Figure 7-B**). If the threshold is

set too high, relevant cells will be excluded from the analysis (**Figure 7-D**) (3,4,11,14,15).

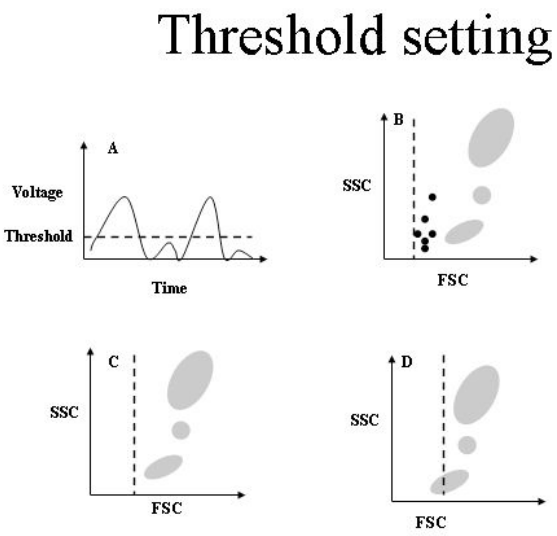


Figure 7. The threshold setting. The discriminator (dash line) has been set on FSC parameter. The example above illustrates that (A) only the signals above the threshold will be recorded (dashed line). In (B) the cells of interest (gray) have been recorded together with some debris and noise (black dots). In (C) the threshold is set correctly and the debris and noise has been excluded. In (D) the discriminator has been set too high resulting in exclusion of some of the cells of interest.

Pulse quantification

Signal processors measure voltage pulses providing numerical values for the total integrated signal, width of the signal and peak height of the signal as pulse area, width and height, respectively (**Figure 8**)

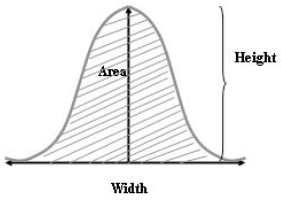


Figure 8. Signal processing results in pulse quantification. The output voltage pulse can be proportional to the width of the signal, the height of the signal, or the integrated area of the signal.

Amplifiers

Flow cytometers are equipped with two types of amplifier, which are capable of amplifying the integrated area of the signal, width of the signal, or the peak of the signal either linearly or logarithmically. The output voltage in linear amplification is directly proportional to the input voltage. Linear amplification is useful for measurement of populations with narrow dynamic range, such as measurement of DNA for cell cycle analysis. The gain of the linear amplifier can be adjusted.

A logarithmic amplifier displays the output voltage proportional to the logarithmic of the input signal. Logarithmic amplification is useful for the measurement of populations with wide dynamic range, as in immunofluorescence. In comparison to a linear amplifier, a logarithmic amplifier has a wider dynamic range and expands the scale of weak signals and contracts the scale of strong signals. For example, at a given linear amplifier setting, two signals with intensities of 100 and 200 will have the same voltage distance away from each other as two signals with intensities of 800 and 900. In contrast to a log amplifier at a given

setting, two signals with intensities of 100 and 200 (twofold increase in intensity) will have twofold longer distance away from each other than two signals with intensities of 800 and 900 (1.125-fold increase in intensity). Most flow cytometers are equipped with four-decade full scale logarithmic amplifier, because this range is useful for several proteins on cell membrane. Cells labeled with fluorochrome-conjugated antibody are normally 10 to 1000 times as bright as unlabeled cells (3,4,11,14,15).

Analogue-to-digital converter (ADC)

The last signal processing step is analog-to-digital conversion. The ADC converts the output of the analog signal into a digital signal for further processing and storage. Most flow cytometers apply 10-bit ADCs, which have 2^{10} or 1024 separate bins or channels and the signals can have a range values from 0 to 1023 (3,4,20,14,15).

1.1.4.2 Emission spectral overlaps

Compensation is a process to correct multi-color FCM data for emission spectral overlaps, which occur between different fluorochromes used for measuring cellular components and represent the biggest problem when performing multi-color FCM analysis (**Figure 9**). This overlap is a consequence of the use of fluorochromes that are measurable in more than on detector. In order to reduce the spectral overlaps, one can use dyes with different absorption optima, which are excited by two or more sequentially positioned laser beams of different wavelengths. As illustrated in **Figure 10**, a cell/particle passes the red laser beam first and then, few microseconds later, the blue laser beam. Thus, the signal excited by the red laser is delayed and separated in time from the blue excited signals. The laser time delay is adjusted to ensure that the signals are contributed to the correct event. However, the majority of the fluorescent dyes suitable for FCM, e.g., Fluorescein isothiocyanate (FITC) and R-

phycoerythrin (RPE), are excited by the same laser line, and fluorescence signals therefore cannot be separated in time. Using suitable optical band pass and long pass filters (**Figure 5**), most of the emission spectral overlaps can be minimized. However, some overlaps still remain (**Figure 9**) and the contribution of signal in detectors not assigned to that fluorochrome must be subtracted from the total signal in those detectors. This process is termed "compensation" and can be shown by two commonly used fluorochromes: FITC and RPE. As shown in **Figure 9** FITC fluorescent signal (green) is detected using a 530/30 nm band pass filter and the RPE fluorescent signal (orange) is measured through a 585/42 nm band pass filter. Both FITC and RPE emit some orange and green fluorescence, respectively, and these signals cannot be eliminated by optical filters. Hence, some of the green fluorescent signals will be detected by the PMT applied for detection of RPE fluorescent signals, whereas the PE fluorescent signals will be detected by the PMT used for detection of FITC fluorescent signals (**Figure 9**).

This undesirable spectral "spillover" (**Figure 11-A**) could be erroneously interpreted, as emission from the orange fluorochrome (RPE) is present on the green fluorochrome (FITC only)-labeled cell population, and the green fluorescent signal is present on the orange fluorescence-labeled (RPE only), as illustrated in **Figure 11-A,** marked **II** and **III,** respectively. The problem of unwanted spectral overlap can be overcome by using either hardware (after signal detection but before logarithmic conversion and digitization) or software (post-collection) compensation or both compensation strategies in a multi-color setup. The leakage of the orange fluorescence signal from FITC is electronically subtracted from the signal detected in orange fluorescence channel (**Figure 11-B, II**) and the leakage of the green fluorescence signal from RPE is electronically subtracted from the signal detected in the FITC channel (**Figure 11-B, III**) (13,16,17).

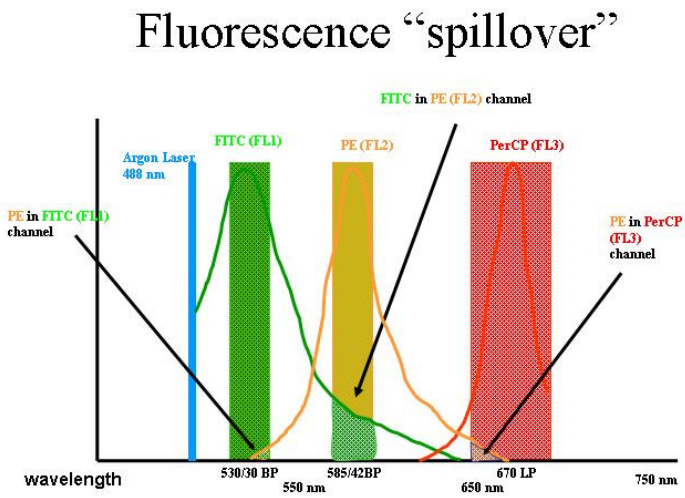


Figure 9. Emission spectral overlaps of FITC, PE and peridinin chlorophyll protein (PerCP).

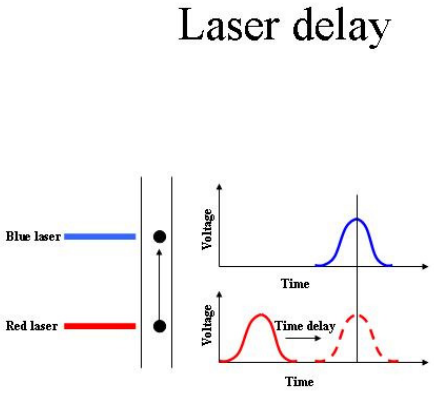


Figure 10. Signal generation in time. A cell is simultaneously labeled with two or more fluorescent dyes that differ in both their absorption optimum and emission optimum, e.g. FITC and allophycocyanin (APC). First, the cell passes the red laser beam and the fluorescent dye APC, with an absorption optimum in the red range of the spectrum, is excited, and will thus emit fluorescence light. The FITC fluorescent

dye is not excited, due to the large difference between the absorption wavelength of the dye and the excitation wavelength of the light source. The red-excited signal from APC is electronically delayed until the same cell passes the second laser beam (blue). At this point FITC is excited, whereas the red fluorescent dye APC is not, because of the large difference in absorption and excitation wavelength. The two signals will arrive at the analysis electronics at the same time and will be detected with separate PMT's

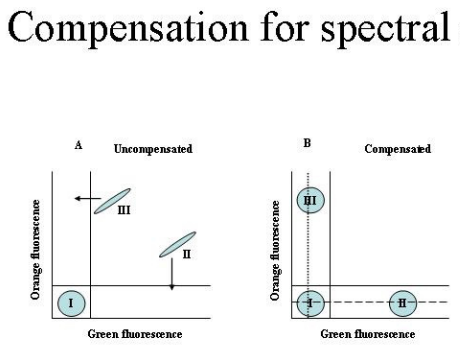


Figure 11. The correction of spectral overlap by electronic compensation. Ellipses and circles represent uncompensated and compensated cell populations, respectively. Cell populations I, II and III are isotype-, green fluorescence- and orange fluorescence-stained, respectively.

In the uncompensated situation (Figure 11-A), one can observe using a green fluorescence vs. orange fluorescence dot plot that the isotype stained (background) cells (I) are located at the lower left quadrant. The green fluorescence positive cells (II) and the orange fluorescence (III) show a diagonal flattened distribution, owing to spectral overlaps of the green fluorescence signal into the orange fluorescence detector and the orange fluorescence signal into green fluorescence detector. Both green and orange spectral fluorescence overlaps (Figure 11-A, II and III) are greater than the background fluorescence of the isotype labeled cells (I).

The correction is made by subtracting the unwanted amount of the green fluorescence signal from the signal in the orange fluorescence detector, as shown in Figure 11-B, marked II. When the mean/median value of the orange fluorescence of green fluorescence-positive cells is comparable to the mean/median value of the orange fluorescence of the isotype stained cells (dashed line), compensation is adequate.

In figure 11-B, marked III, the unwanted contribution of the orange fluorescence to the green fluorescence

detector (III) is corrected. The mean/median value of the green fluorescence of the orange fluorescence-positive population is comparable to that of the green fluorescence of the isotype stained cells (dot line).

1.1.4.3 Data display

The list mode data can be analyzed applying a computer and software. The software is often part of the same computer system used to control the instrument during acquisition of the specimens. External software provided by a third-party vendor can be used for data analysis, often with the possibility of post-collection compensation feature. The data presentation can be performed in different way by using various displays, including univariate histograms or correlated dot plots. A univariate histogram is often used for visualization of a single parameter FCM data. Cell number is displayed against intensity of the measured parameter (**Figure 12**).

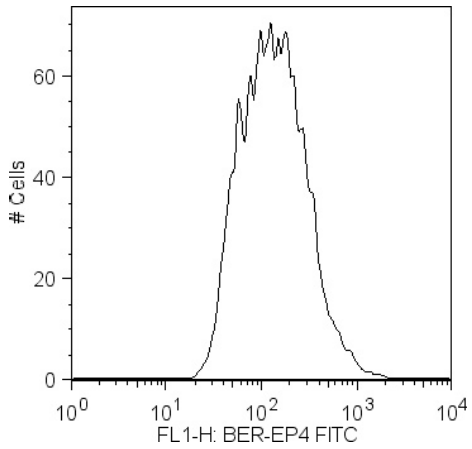


Figure 12. A histogram showing fluorescence against cell number. The signals from a wide distribution of fluorescence intensities are depicted on a logarithmic scale. The wide distribution reflects biological variability within a population.

When two parameters are visualized at the same time, a correlated dot plot is applied; each single cell is depicted as a dot on the screen and shows the value of the two parameters measured (**Figure 13**).

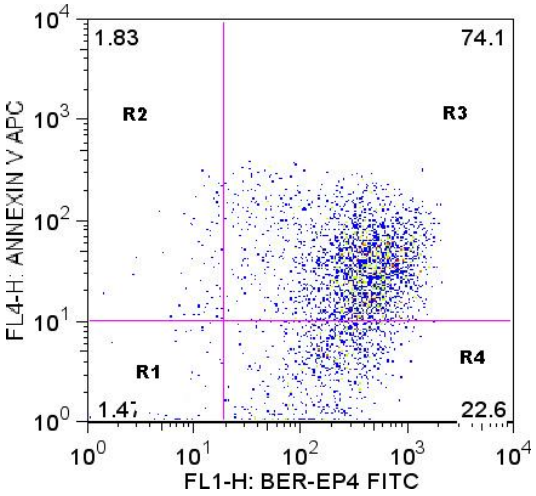


Figure 13. Flow cytometers enable the simultaneous measurement of different parameters. Each dot on the quadrant R3 represents one cell that is labeled with Ber-Ep4 and Annexin-V-conjugated to a green fluorescence signal (FL1=FITC) and a far red fluorescence signal (FL4=APC), respectively.

1.2. Ovarian cancer

1.2.1. Epidemiology, etiology and pathogenesis

Ovarian cancer is the eighth most commonly diagnosed cancer and the seventh leading cause of cancer death in women worldwide, comprising nearly 4% (225,500) of the total new cancer cases and 4% (140,200) of the total cancer deaths among females in 2008 (18). The incidence rates in economically developed countries are nearly twice as in economically developing counties, and mortality rates are 2% higher in more economically developed compared to less economically developed counties (18). From 1999-2008, a total of 4,570 new cases of ovarian cancer were recorded in Norway and the age-adjusted incidence rate ranged from 10.8 to 13.8

per 100,000 person-years (19). Despite moderate improvement, the prognosis of this disease is still poor, with a 5-year relative survival at about 44% in Norway in 2008 (19). The high fatality rate is mainly attributed to diagnosis at advanced stage (III-IV) based on the 1988 International Federation of Gynecology and Obstetrics (FIGO) staging system (20). The majority of ovarian cancers are epithelial (ovarian carcinoma; OC) and afflict women near menopause or post-menopause, median age at diagnosis being between 60 and 65 years. Approximately 10% of women with OC have positive family history, whereas the remaining 90% of cases are sporadic (21).

Although the origin and evolution of OC remain largely unknown, several risk factors, including age, ovulation, reproductive history and genetic polymorphisms have been proposed to be predisposing factors (21-25). Age is an important risk factor for OC, as reflected in the high incidence of the disease in older women. Incessant ovulation, early age at menarche, late age of natural menopause and nulliparity are associated with increased risk for this disease. In contrast, there is evidence that the use of oral contraceptives and pregnancy reduce the risk of ovarian cancer considerably (21-25).

Genetic abnormalities such as *TP53* mutation with loss of p53 function are frequently found in both sporadic and familial OC cases. In addition, inheritance of DNA repair defects and mutations in the *BRCA1* and *BRCA2* genes increase the risk of developing this disease (21-26).

Recent morphologic and molecular genetic studies have suggested that OC develops *de novo* and that based on distinctive morphologic and molecular features, OC may originate from the fallopian tubes, uterus, cervix, and upper vagina which develop from the Müllerian duct of the embryo. Thus, OC were divided in two groups, designated type I and type II tumors (22,27-

29). Type I are slow-growing tumors which arise from borderline tumors and include low-grade serous, low-grade endometrioid, clear cell, mucinous and transitional carcinomas. These tumors are genetically stable and lack *TP53* mutations, although frequent mutations in the *KRAS*, *BRAF*, *ERBB2*, *PTEN*, *CTNNB1* and *PIK3CA* genes occur. Conversely, type II tumors, comprising high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas, are fast-growing, highly aggressive tumors and often present at advanced stage at diagnosis. They are identified by genetic instability and display *TP53* mutations in more than 80% of cases, as well as *BRCA* mutations and *CCNE1* amplification, but rarely harbor the mutations observed in type I tumors (22,27-29). These findings may suggest that each type of OC develops along different molecular pathways. Other authors have suggested that the five major subtypes of OC (high-grade serous, clear cell, endometrioid, mucinous and low-grade serous), which have different biomarker expression, are distinct disease entities (30).

Until recently, it was widely accepted that OC arises in the ovarian surface epithelium or cortical inclusion cysts. Lately, this view of ovarian carcinogenesis has been challenged through morphological, immunohistochemical and molecular genetic studies suggesting that low-grade and high-grade serous ovarian carcinomas derive from the fallopian tube epithelium implanting on the ovary (29,31,32).

1.2.2. Clinical features, classification and staging

OC is an aggressive disease with a high mortality rate that reflects the fact that this tumor develops without an obvious symptom profile, rendering it difficult to diagnose at early stages and resulting in widespread disease at diagnosis. The symptoms of ovarian cancer are often vague and include abdominal pain or discomfort, bowel irregularity, persistent fatigue, weight loss, distended or tense abdomen, pain outside the abdominal cavity, frequent urination, and

respiratory difficulties (21,27,33). Women suffering from one or more of the aforementioned symptoms are likely to be referred to an internal medicine or general surgery unit instead of a gynecological unit, unlike women with symptoms such as vaginal bleeding or discharge, resulting in delayed diagnosis and treatment (33). During examination, physical findings typically include a palpable ovarian mass, in addition to an increase in abdominal girth as a result of ascites formation and dyspnea due to pleural effusion (21,27,28). Transvaginal ultrasonography (TVS) or computed tomographic (CT) is often carried out for further assessment of the pelvic mass, if OC is suspected on the basis of the above-mentioned findings. The presence of a complex ovarian mass with both solid and cystic components, sometimes with septations and internal echoes, is highly suggestive of OC. The occurrence and formation of ascites is suggestive of advanced disease and an exploratory laparotomy is usually done for histological confirmation, staging and tumor debulking (21,27,28).

The symptoms of OC are often nonspecific. Thus, early detection might to a large extent improve survival, if discovery of the early lesions on routine physical examination can lead to surgery prior to metastasis and if tumors remain localized for a satisfactory interval to allow effective screening (21,27). Given its prevalence, strategies for early recognition of this disease must have a high sensitivity (>75%) for early-stage disease, and very high specificity (99.6%) to accomplish a positive predictive value of 10% or greater (21,27). Serum cancer antigen-125 (CA-125), TVS and a combination of both methodologies have been evaluated for their ability to detect OC at early stages. However, CA-125 level as a single marker in the screening test lacks sensitivity and specificity. Greater specificity can be achieved by combining CA-125 and TVS, by monitoring of CA-125 concentration over time, or both. The use of CA-125 with additional biomarkers in a panel might increase the sensitivity.

Development of sensitive and specific biomarkers based on different proteomic profiles has been suggested to be effective in early detection of OC (27).

Classification of primary cancers of ovary is performed according to the structures of the ovary from which they are derived. Ovarian neoplasms are classified as surface epithelial tumors, sex cord-stromal tumors, germ cell tumors and secondary tumors (34). Most of the epithelial tumors are believed to develop from epithelial cells covering the ovary or lining inclusion cysts (21,34), and these cells are thought to derive from the coelomic epithelium of mesodermal origin. OC are the most common ovarian malignancies and account for 90% of all malignant ovarian neoplasms. OC is histologically categorized according to cell type into serous, mucinous, endometrioid, clear cell, transitional, squamous, mixed and undifferentiated (35,36). According to world Health Organization (WHO) classification based on microscopic evaluation of the amount of epithelial cell proliferation, the degree of nuclear atypia and the presence or absence of stromal invasion, tumors in each of these categories are further divided into benign, borderline and malignant forms (35,36). Serous tumors are the most frequent epithelial tumors, and are composed of cells ranging in appearance from those resembling the fallopian tube epithelium in well differentiated tumors to anaplastic cells in poorly differentiated tumors (34,35). The growth pattern of serous carcinoma varies from glandular to papillary to solid. OC is histologically graded 1 to 3, corresponding to well differentiated, moderately differentiated and poorly differentiated, respectively (35,37).

OC spreads primarily by direct shedding of carcinoma cells throughout the peritoneal cavity forming ascites and multifocal tumor metastases to other peritoneal compartments/organs, including the urinary bladder, uterus, bowel, omentum, and mesocolic lymph nodes. The most frequent extra-abdominal site of distant metastasis is the pleural space, and distant metastasis

to the parenchyma of the liver, lungs, and other organs is less common (27). Patients with early-stage OC (stages I-II) have long-term survival (>10 years) rates in the 80-95% range, whereas 75% of patients with advanced disease (stages III-IV) have low long-term survival rates, ranging from 10-30% (27). The spreading pattern of OC is reflected in the FIGO staging system, detailed in **Table 1** (37).

Table 1. FIGO staging of OC (1988) (37)

Stage I	**Growth limited to the ovaries**
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact
Ic[a]	Tumor either Stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage II	**Growth involving one or both ovaries with pelvic extension**
IIa	Extension and/or metastases to the uterus and/or tubes
IIb	Extension to other pelvic tissues
IIc[a]	Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage III	**Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equals Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum**
IIIa	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery
IIIb	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes
Stage IV	**Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV.**

[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings or ascites

1.2.3. Treatment of OC

Surgical treatment of OC may be adequate if the tumor is well-differentiated and confined to the ovary. For patients with advanced-stage OC, a combination of surgery followed by adjuvant chemotherapy is standard therapy. The type of surgical treatment depends upon disease stage, as well as tumor type and grade. Young women with early-stage (stage I), low-grade disease, who wish to preserve their fertility, are treated by removal of the involved ovary (unilateral oophorectomy) (21,27,28). In advanced-stage disease, the surgical procedure includes a total abdominal hysterectomy, bilateral salpingo-oophorectomy along with examination of all peritoneal surfaces, omentectomy, peritoneal biopsies and retroperitoneal assessment involving both the pelvic and para-aortic area, as well as drainage of ascites or peritoneal washing (21,27,28). The aim of the initial surgical effort is to achieve optimal debulking. The most widely-accepted definition of optimal debulking has been residual tumor <1 cm in diameter (27,38). Several reports have shown that the volume of residual disease remaining after cytoreductive surgery inversely correlates with survival (reviewed in 38). In recent years, the definition of optimal debulking has changed to also include maximal debulking efforts, with the end goal of complete resection of all visible disease (27,38). On the basis of retrospective studies, the suggestion for complete resection of all visible disease is becoming more widely appreciated, as it has been documented to improve overall survival (OS) (38).

In the majority of cases, adjuvant chemotherapy is a standard postoperative treatment (21,27,28). The first-line chemotherapy regimen for OC consists of paclitaxel in combination with a platinum-based compound (cisplatin or carboplatin) or platinum-based therapy only. In large randomized clinical phase III trials of advanced-stage OC, using cisplatin vs. carboplatin in combination with paclitaxel resulted in comparable results in term of patient survival.

However, a combination of carboplatin-paclitaxel was the preferable regimen due to less toxicity, superior quality of life, and easier administration (39-41). Despite the improvements in outcome provided by these therapy regimens, the preponderance of the women with advanced-stage OC will relapse and eventually die of their disease (41). As new chemotherapy compounds have been recognized and shown activity for recurrent disease, several approaches have been established to determine if additional cytotoxic agents such as gemcitabine, methoxypolyethylene glycosylated liposomal doxorubicin and topotecan in primary treatment would further improve progression-free survival (PFS) or OS. Interim analysis provided no benefit in survival of the patients (42-44). Hence, carboplatin-paclitaxel combination remains the standard front-line therapy.

Since attempts to improve patient survival by adding other agents to the standard front-line therapy have yielded disappointing results, Katsumata et al. (45) have addressed the frequency of the treatment by performing a randomized phase III trial with dose-dense regimen of a weekly paclitaxel in combination with carboplatin every three weeks. Significant improvement in PFS and OS survival using this regimen was observed compared to the conventional regimen. However, toxicity related to hematological side effects was higher in the dose-dense group.

Intraperitoneal (IP) treatment with platinum-based agents and taxanes has been shown to be a valuable regimen for standard care for advanced OC based on the leverage of chemotherapeutic agents and the delivery of high doses into the abdominal compartment in an attempt to maximize drug activity against OC (46-48). A number of studies have shown a benefit in terms of prolonged patient survival of IP treatment compared to single intravenous (IV) administration of chemotherapy for OC Patients (49-51). Armstrong et al. evaluated the efficacy of palitacxel and cisplatin as either an IV regimen only or as an IV/IP combination

for six cycles in optimally debulked stage III OC patients. Despite the fact that only 42% of patients in the IP group completed the six cycles of treatment, significant gain in PFS and OS was confirmed. The median PFS was 23.8 months in the IP arm and 18.3 months in the IV arm, whereas the OS for the IP arm and IV arm was 65.6 months and 49.7 months, respectively. Although this study revealed increased toxicity and reduced quality of life during treatment, the authors advocated the use of IP chemotherapy regimen as standard care for first-line treatment (46). However, the application of IP chemotherapy regimen as first-line treatment has been hotly debated due to the toxicity, complication rate, methodological difficulties and variability in the study design related to IP drug administration (47,52-55).

Neoadjuvant chemotherapy (NACT), defined as administration of platinum and taxane-based chemotherapy prior to debulking surgery, is playing an important role in patients with advanced OC for whom initial surgical treatment is not feasible (38,56). Reduction in morbidity applying NACT followed by interval debulking compared to initial cytoreductive surgery in advanced OC has been reported. However, OS rates are similar between the two treatment groups (38). The preliminary results of a large phase III trial (EORTC 55971) suggested that NACT followed by interval cytoreduction vs. primary cytoreduction were comparable in stages IIIC and IV disease and the OS rates were 30 and 29 months, respectively (56,57). In addition, patients assigned to neoadjuvant chemotherapy have no significant benefits related to survival, adverse effects, quality of life, or postoperative morbidity or mortality compared to primary cytoreductive surgery (57). The basis for the recommendation of the NACT followed by interval cytoreduction as an adequate routine therapy strategy for advanced OC has been challenged because current data are controversial and since this treatment strategy has no potential to improve PFS or OS. Hence, primary

optimal cytoreductive surgery remains the recommendation for the management of advanced OC (38,58).

Despite improvement in surgical and chemotherapeutic strategies, the majority of women with advanced OC will experience disease recurrence and resistance to therapy. Hence, exploration of other treatment modalities, including targeted therapy, has been evaluated in multiple studies in recent years. Potential strategies for improving outcome and overcome resistance in OC include the inhibition of signal transduction pathways and targeting DNA repair. The most promising agents are the antiangiogenic agents, including monoclonal antibodies to VEGF ligand (bevacizumab), small molecule tyrosine kinase inhibitors (TKIs) targeting the VEGF pathway and soluble decoy VEGFR (aflibercept), in addition to monoclonal antibodies against epidermal growth factor receptor (EGFR) (59-61). The results of clinical testing of some of the targeted agents are mixed. Evaluation of the EGFR inhibitors revealed disappointing results, whereas the VEGF inhibitor bevacizumab showed promising results as single-agent in phase II trials in recurrent OC (59-61). Due to the positive results form these bevacizumab trials, GOG (GOG218), International Collaborative Ovarian Neoplasm (ICON7) and OCEANS began multiarm Phase III trials focusing on the application of bevacizumab in front-line therapy (61,62). GOG218 was a 3-arm placebo controlled study where the patients received carboplatin + paclitaxel + placebo (arm1) versus carboplatin + paclitaxel + bevacizumab followed by placebo (arm 2) versus carboplatin + paclitaxel + concurrent and extended bevacizumab (arm 3). The ICON7 was a 2-armed trial without placebo for OC patients at all stages (61,62). Preliminary data from the GOG218 trial demonstrated a significant improvement in the median PFS, 14.1 months versus 10.3 months in patients treated with concurrent and maintenance bevacizumab arm and the placebo arm, respectively. The median OS for the 3 arms was 39.3 months, 38.7 months and 39.7 months, respectively (61,62). Recent data for the ICON7 trial confirmed the findings of GOG218 in

term of PFS, but the data are immature regarding OS (61,62). OCEANS is a placebo-controlled study comparing carboplatin and gemcitabine with or without bevacizumab in recurrent disease, and results are at present not available (62). Several potential targets in OC including folate receptor alpha (FRA), poly (ADP-ribose) polymerase (PARP), aurora kinase and mTOR are currently under evaluation (48).

1.2.4 Clinicopathologic and molecular prognostic factors

Despite increased knowledge regarding the etiology of OC, as well as the use of aggressive surgery and chemotherapy with newly developed drugs, there has been a minor change in the OS of OC patients. The prognosis of OC can be correlated with various clinicopathologic factors, including tumor stage, tumor grade, histological type, size of residual disease, age, the presence of ascites, and serum CA-125 levels (21,63-68). Tumor staging is an attempt to stratify patients into different prognostic groups based on the extent and volume of disease at diagnosis and it remains one of the most significant predictors of patient outcome. The FIGO classification (**Table 1**) is still the most commonly used staging guideline, and correlates well with patient outcome (21,27,37,65,66).

A large number of molecular markers have been identified and suggested to be involved in the development and progression of OC, and serve as potential prognostic factors and therapeutic targets. These include proteins and genes implicated in cell proliferation, motility, angiogenesis, apoptosis, chemoresistance, and chromatin maintenance (68-72). DNA ploidy has been reported to be of independent prognostic value in OC (73-75). The EGFR family receptors, especially EGFR and HER2, are overexpressed in OC, and have been associated with advanced disease, chemoresistance and worse prognosis (61,76). Alterations in the tumor suppressor gene *TP53* are the most frequent molecular events reported in OC (77), and the

relationship between mutations of *TP53* and OC patient survival has been investigated in many studies. However, no obvious association with worse prognosis has been found (69,71,77). Numerous reports have investigated the possible roles of *BRCA1* and *BRCA2* mutations with respect to prognosis and survival in OC, but results have been inconclusive. Some of these reports have shown a more favorable survival for OC patients who are carriers of *BRCA1* and *BRCA2* mutations compared to non-carriers, whereas other studies did not find a survival benefit for these patients (reviewed in 71). High serum Human Epididymis protein 4 (HE4) level has been reported to be a strong and independent indicator of worse prognosis (78,79). Among the numerous other markers that have been suggested to a have prognostic value in OC are cancer-associated molecules of the integrin, matrix metalloproteinase (MMP) and Ets family (80), HBXAP (Rsf-1), NF-κB, Fatty acid synthase, Apo-E, Mesothelin, Claudins, Kallikreins, Twist and Snail (68,72,81), cytokines, VEGF and cell cycle- and apoptosis-regulatory proteins (68), as well as the epigenetic modifications which involve DNA methylation, histone modifications and dysregulations of nucleosomes and miRNA (70).

1.3. The serosal cavities

1.3.1. Malignant effusions

The embryonic coelomic cavity gives rise to the pleural, peritoneal and pericardial serous cavities. The term *serous* refers to the small amount of serum-like fluid each cavity contains. Under normal conditions the cavities are collapsed sacs invaginated by the lungs, heart or intestines. Each cavity is completely closed, except for the peritoneal cavity at the point at which it receives the fimbriated ends of the fallopian tubes. The outer layer of each serous cavity is the parietal layer, and the inner layer, which is directly in contact with the enveloped organ, is the visceral layer. The layers, which are separated by a thin film of fluid, are in

contact with each other. Thus, under normal conditions, each cavity is only a potential cavity. Each cavity is lined by a monolayer of mesothelial cells beneath which is a layer of connective tissue, supplied with blood vessels, lymphatics and nerves (82-84).

The appearance of a malignant effusion within the serosal cavities is a frequent clinical manifestation of advanced cancer and is a poor prognostic indicator (84). It usually affects the peritoneal or pleural space, with less frequent involvement of the pericardial space. The formation of malignant ascites is usually caused by ovarian, endometrial, breast, gastric, colorectal, lung, esophageal, pancreatic, hepatobilliary and primary peritoneal carcinomas (84-86). The development of malignant ascites may be influenced by factors such as lymphatic obstruction by tumor cells, immunomodulators, enhance vascular permeability, and increased production of fluid by cells lining the cavities (84,85,87). The most common symptoms reported by the majority of patients with malignant ascites are abdominal swelling, pain, nausea, anorexia, vomiting and fatigue (84,87,88). Treatment of malignant ascites is multidisciplinary. However, paracentesis and diuretics remain the mainstay of management of patients with malignant ascites, followed by several other modalities, such as peritoneovenous shunts, dietary measures, permanent drains, investigational therapies and IP chemotherapy (85,87,88).

There are several etiologies for pleural effusions, with inflammatory/infectious causes and malignancy being the most common causes of exudative effusions (84,89-91). Lung and breast adenocarcinoma (AC) are the most frequent malignant diseases, followed by gastric AC, OC, malignant mesothelioma (MM) and lymphoma (84,89-91). Lymphatic obstruction, hematogenous spread, microvascular permeability, angiogenesis and chemotaxis have been suggested to contribute to tumor growth and the formation of malignant pleural effusions (91,92). The prognosis of patients with malignant pleural effusions is associated with various

factors, including effusions volume (93), tumor type, and biochemical characteristics of the pleural fluid, such as pH and the concentration of protein and lactate dehydrogenase (LDH) (94). The median survival of patients with malignant pleural effusion after clinical diagnosis ranges between 4 to 8 months (93-95). Dyspnea is the first and commonest presenting symptom in patients with malignant pleural effusion (90). Other symptoms are cough, chest pain and heaviness (90). Several treatment strategies, including thoracocentesis, pleurodesis, fibrinolytics, pleurectomy, chemotherapy, pleuroperitoneal shunt and chest tube drainage, can be applied (90,96,97).

While the biology of the different cancers affecting the serosal cavities and the clinical significance of effusions may differ, the presence of carcinoma cells in a peritoneal and/or pleural effusion indicates advanced stage disease and metastasis beyond the primary organ, and is associated with significant diagnostic and prognostic implications (84,86,90). The microenvironment in effusion specimens is unique and different from their corresponding primary tumor and other organs (84,86,98,99). Primary tumors are composed of two interactive compartments, parenchyma and stroma, in which tumor cells constitute the parenchyma, whereas the stroma is the surrounding connective tissue composed of various elements, including vasculature, fibroblasts, inflammatory cells and extracellular matrix (99-101). Recent evidence indicates that the stroma is important for tumor maintenance, growth, invasion and metastasis (99-101). In contrast, mesothelial cells and leukocytes, and in the case of cancer, malignant cells, are the main constituents in the microenvironment of malignant effusions, with the absence of stroma and vasculature, resulting in a hypoxic microenvironment with reduced nutrient and oxygen supply. These floating cancer cells have a unique ability to proliferate and to progress despite the unfavorable environment and the lack of solid-phase scaffolding seen in primary tumors. The ability of malignant cells in

effusion to proliferate and survive may suggest their skill of acquiring nutrients, growth factors and other proteins produced by mesothelial cells and inflammatory cells or cancer cells themselves, contributing to disease progression through their unique phenotype (84,86).

The cytological diagnosis between cancer cells, inflammatory cells (notably macrophages) and reactive or malignant mesothelial cells can at times be complicated. The morphological changes of reactive mesothelial (RM) cells caused by various stimuli such as injuries, chemotherapy or radiation, may give rise to severe cytoplasmic and nuclear alterations, resembling malignant cells (82,84,102). Furthermore, macrophages constitute a significant cell population in effusion specimens, resulting in possible confusion with cancer cells (103). Metastatic AC may be difficult to differentiate from MM. Thus, different methods such as immunohistochemistry (IHC), electron microscopy, FCM, polymerase chain reaction (PCR), immunofluorescence, telomerase activity, fluorescence in situ hybridization (FISH) and DNA ploidy analysis have been applied as adjuncts to morphology with the aim of increasing the diagnostic accuracy (84,86). IHC on cytospin specimens, formalin-fixed cell block sections, smears and liquid-based samples is the most widely used ancillary method in this setting, and has benefited from continuous improvement and expansion of the antibody panels available (84,85,104-106). Difficulties are commonly encountered due to the overlapping morphological characteristics of AC and MM or RM cell populations (86,106). The evaluation of the performance of various antibodies in differentiating between these entities is made complicated due to the fact that most of the reports have analyzed AC of different origins. IHC markers proposed to be valuable in differentiating between AC and MM are summarized in **Table 2** (84,102,104,106-119).

Recently, high-throughput molecular methods, including microarrays, proteomics, and comparative genomic hybridization (CGH) have been applied in this context (84,106).

Effusion specimens are suitable for FCM analysis, since they consist of single cells or small cell clusters. In effusion cytology, several investigators have applied FCM to simultaneous measure DNA aneuploidy (120-125) and expression of surface makers (124,125) to detect malignant cells, sometimes in combination with immunophenotyping of admixed lymphoid cells (126,127). The basis for biological studies is a reliable characterization of the cells in effusions. Our group has previously studied the potential of FCM immunophenotyping of epithelial and mesothelial cells in effusions in the diagnostic setting, and have subsequently applied FCM to investigate the expression of cancer-associated molecules in effusion cytology (84). However, the potential of FCM as a tool for the analysis of biological parameters (e.g., cell proliferation, cell signaling and apoptosis) in clinical specimens is still unfulfilled. The use of FCM in this setting requires further improvement in parameters such as instrument settings, panel selection and specimen handling.

Table 2. Summary of proposed IHC markers in effusion diagnosis (84,102,104,106-119)

Markers	**Target Cell**	**Sensitivity (%)**	**Specificity (%)**
Ber-EP4	AC	64-100	60-100
B72.3	AC	35-98	42-100
Leu-M1 (CD15)	AC	30-100	51-100
MOC-31	AC	67-100	76-100
BG-8	AC	73-100	91-100
CEA	AC	0-100*	41-98
MUC4	AC	96	98
CA19-9	AC	27-86	97-100
Claudin-3	AC	86	96
TTF-1	AC	58-100	39-100
CA125	AC	95-100	12-34
Tenascin-X	MM	92	89
Calretinin	MM	58-100	62-100
Podoplanin (D2-40)	MM	85-100	42-100
CK5/6	MM	53-100	57-89
Thrombomodulin	MM	33-100	33-92
HMBE-1	MM	84-98	0-80
WT1	MM	43-100	0-100
Mesothelin	MM	75-100	61-100

* Using both polyclonoal and monoclonal antibodies

1.3.2. Molecular alterations in malignant effusions

Our group has reported on the differential expression of a large number of cancer-associated molecules in cancer cells in effusions compared to primary tumors and solid metastases. These molecules have biological functions related to tumor development, progression and disease outcome, and include tight junction proteins (claudins), cell adhesion proteins (cadherins and integrins), growth factors and their receptors (neurotrophin receptors, VEGF, and granulin-epithelin precursor; GEP), proteolytic enzymes (MMP) and microRNAs (miRNAs). Additionally, a large number of molecules have been found to be associated with OS and PFS in effusions, including the insulin-like growth factor (IGF) pathway, cytokines and chemokine receptors, signaling molecules (mitogen-activated protein kinases; MAPK, phosphatidylinositol-3-kinase pathway; PI3K), mitotic regulators (cyclins) and transcription factors (ETS family members, E-cadherin repressors) (72,84). These studies emphasize the need to study effusion specimens for their biological and clinical relevance.

1.4. Apoptosis

1.4.1 The apoptotic signaling networks

Apoptosis or programmed cell death is a highly regulated process of cell deletion and plays an important role in the development and maintenance of cellular homeostasis. This process is characterized by typical morphological characteristics including cell membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation, finally ending with the engulfment by macrophages, dendritic cells or neighboring cells, thereby avoiding an inflammatory response in the surrounding tissue. Apoptosis is well balanced and regulated in a physiological context, and failure of this mechanism may result in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer (128-132). The characteristic morphological features that define apoptosis are mediated by caspase

activation and cleavage of specific cellular proteins within a cell (128,132). The apoptotic signaling pathways are divided in two major categories, namely the extrinsic, or death receptor-mediated, and the intrinsic, mitochondria-mediated pathways (**Figure 14**). In addition, other pathways also exist, such as the granzyme B-mediated and the endoplasmic reticulum (ER)-mediated pathways.

The extrinsic pathway requires active participation of members of the tumor necrosis factor (TNF) superfamily of receptors (see below) and their ligands. Receptor-mediated apoptotic signaling is initiated by ligation of transmembrane death receptors to activate membrane-proximal (activator) caspase-8 and -10, which in turn cleave and activate executor caspases such as caspase-3 and -7 or Bcl-2 family proteins, depending on the cell type (130-136).

The intrinsic pathway is initiated by a number of factors, including DNA damage, hypoxia, survival factor deprivation, radiation, chemotherapy and kinase inhibitors. Upon disruption of the outer mitochondrial membrane, a set of mitochondrial proteins, including cytochrome c, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (Smac/DIABLO), Omi/HtrA2, apoptosis inducing factors (AIF) and endonuclease G, is released into the cytoplasm. Once in the cytosol, these apoptogenic proteins mediate the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors. The release of cytochrome c forms a complex with the adaptor protein Apaf-1 and pro-caspase-9, resulting in activation of pro-caspase-9. The binding of pro-caspase-9 to the apoptosome forms the caspase-9 holoenzyme that cleaves and activates the downstream caspases, such as caspase-3, resulting in substrate cleavage and the appearance of apoptosis-related morphological changes (**Figure 14**). Despite the difference in the initiation of both the extrinsic and intrinsic pathways, crosstalk between the two pathways at both the executioner and initiation levels has been reported (129,132-138).

In addition, cellular Ca^{2+} overload or perturbations of intercellular compartmentalization (139), DNA damage (140), and the lysosomes (141) have been shown to participate in apoptotic signaling.

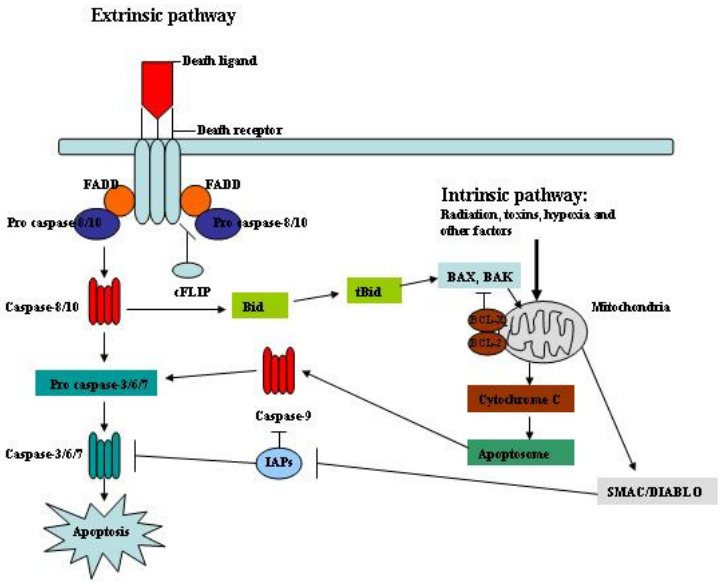


Figure 14. Scheme of extrinsic and intrinsic apoptotic pathways.

1.4.2. Regulation of apoptosis

The apoptotic machinery, both extrinsic and intrinsic, is regulated by several molecules, such as Bcl-2 proteins, p53, RAS, Smac/DIABLO, c-FLIP, NF-κB, inhibitor of apoptosis proteins (IAPs), heat shock proteins (HSPs), protein kinases and the PI3K pathway members (132,133,136,142). The Bcl-2 family of proteins are the most important regulators of the intrinsic pathway, including both pro-apoptotic members such as Bax, Bak, Bok, Bad, Bmf,

Bcl-Xs, Bid, Bik, Bim, Noxa, Puma and Hrk, which promote the release of mitochondrial cytochrome c, and antiapoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1, which act as repressors of apoptosis by blocking the release of mitochondrial cytochrome c (133,142,143). Regulation of the caspase cascade requires involvement of IAPs, which upon binding with caspases cause a steric block, resulting in inhibition of caspase-substrate binding (135,144,145).

1.4.3. Dysregulation of apoptosis in cancer

It is now evident that defects in the apoptotic pathways contribute to tumor initiation, progression, metastasis, and treatment failure (132,133,136,146,147). Among the various mechanisms for resistance to apoptosis is the inactivation of pro-apoptotic genes or overexpression of anti-apoptotic genes. Overexpression of Bcl-2 in follicular B-cell lymphoma, caused by the chromosomal translocation t(14:18), has been shown to cooperate with the oncoprotein c-MYC in suppressing apoptosis, thereby contributing to tumorigenesis in lymphomas and various cancers, and is associated with severity of malignancy and resistance to different chemotherapy drugs and radiation (147,148).

Hypoxia is a frequent phenomenon in various solid tumors and the key regulator of hypoxia-induced apoptosis is hypoxia-inducible factors (HIFs), which combined with other factors can either induce or inhibit apoptosis (149,150). A large number of studies have shown that loss-of-function of different tumor suppressor genes or oncogene gain-of-function contributes to increased levels of HIFs (HIF-1α and HIF-2 α), leading to tumor aggressiveness, metastasis promotion and patient mortality. These observations reveal the consequence of deregulation of apoptosis in tumor progression (149-153).

A large number of genes and molecules reported to be involved in the either induction or inhibition of apoptosis, including miRNAs, p53, the PI3K pathway, Apaf-1, IAPs, cFLIP,

Bcl-2 and Bcl-X_L, have been reported to be aberrantly regulated or overexpressed in OC, resulting in chemoresistance (reviewed in 154-157).

1.5. Death receptors

1.5.1. The death receptor family

Death receptors (DR) belong to the tumor necrosis factor receptor superfamily (TNFR) and consist of TNFR1, Fas/CD95, DR3, the TNF-related apoptosis-inducing ligands (TRAIL) receptors DR4 (TRAILR1) and DR5 (TRAILR2), DR6, ectodysplasin A receptor (EDAR) and the low-affinity nerve growth factor receptor $p75^{NGFR}$. These receptors contain a cytoplasmic region of approximately 80 amino acids termed the death domain (DD). Once DR are activated by corresponding ligands, the DD recruits a number of molecules that mediate both death and proliferation of the cells. These molecules in turn recruit other molecules via their DD, followed by activation of caspases resulting in destruction of the cell. Decoy receptors counting TRAILR3 (DcR1), TRAILR4 (DcR2), DcR3 and osteoprotegrin (OPG) do not possess DD and hence are not able to transmit apoptotic signals upon binding to death ligands (146,158,159).

1.5.2. Death receptor signaling

Two types of DR signaling complexes have been described. The death-inducing signaling complex (DISC) induced by TNFR1 is different from the DISC induced by CD95 or DR4 and DR5. The DISC complexes induced by CD95, DR4 and DR5 consist of oligomerized/ trimerized receptors, the DD (Death Domain)-containing adaptor protein FADD (Fas-Associated Death Domain), procaspase-8, procaspase-10 and the cellular FLICE-inhibitory protein ($FLIP_{L/S}$). Activation of procaspase-8/10 at the DISC by cleavage leads to the

activation of downstream effector caspases (caspase-3,-6 and 7) and apoptosis. Two different pathways can be activated depending on the DR and cell type (158-160).

Stimulation of TNFR1 results in the formation of two complexes. Complex I is assembled at the membrane and includes TNFR1, TNF, RIP (receptor-interacting protein), TRADD (TNFR-associated death domain protein) and TRAF-1/2 (TNFR-associated factor), which is responsible for activating the NF-κB signaling pathway. Due to the lack of FADD and procaspase 8, complex I is dissociated from TNFR1 and translocated to the cytosol in order to bind FADD and recruit caspase 8/10 forming complex II that activates downstream death signaling. Upon successful activation of NF-κB by complex I, the cellular level of $FLIP_L$ increases, resulting in blockage of apoptosis and promoting of cell survival (158-160) (**Figure 15**).

Upon ligand binding, DRs preferentially induce apoptosis in cancer cells. However, tumor cells can flee DR-induced apoptosis through several mechanisms, such as overexpression of decoy receptors, alterations of antiapoptotic or prosurvival molecules, dysfunctional DISC components, mutation of the receptors, increased expression of IAP proteins and increased expression of antiapoptotic miRNAs (159,161,162).

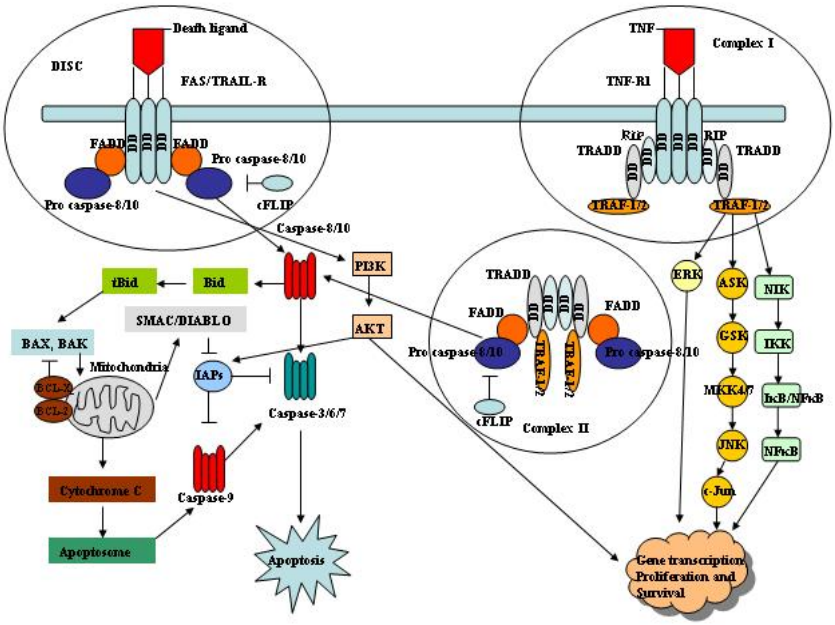


Figure 15. Two types of DR signaling complexes.

1.5.3. Non-apoptotic DR signaling

Several studies have demonstrated that DR and their ligands are not implicated only in promoting cell death but also in promoting cell survival and proliferation (163-166), pro-inflammatory activity (167-169) and tumor growth and progression (169-172). These non-apoptotic signaling activities are mediated by NF-κB, the MAPK member ERK, and AKT (163-172) (**Figure 15**).

1.5.4. Resistance of OC cells to DR-mediated apoptosis

The current treatment regimen (cisplatin or carboplatin coupled with paclitaxel) of OC renders cancer cells to undergo apoptosis. However, the biggest hurdle in the treatment of OC is resistance against currently applied drugs observed in patients. Although the underlying

mechanisms of this resistance are not fully clear, growing evidence suggest deregulation of or defects in apoptotic pathways, as well as activation of survival pathways (155,162,173). A number of studies have demonstrated variable sensitivity to TRAIL-induced apoptosis in OC cell lines (174-179). In addition to lack of or reduced DR expression (177, 180,181), cFLIP and XIAP levels have been implicated in chemoresistance as well (176-179). Increased turnover of caspase-3 may be the underlying cause of inducible TRAIL resistance in previously sensitive OC cells (182), and activation of PI3K by malignant ascites fluid conferring TRAIL resistance by increased cFLIP level was documented (183). Alternatively, IL-8 was shown to inhibit TRAIL-induced apoptosis via the p38-MAPK pathway (184). CD95-induced apoptosis in a wild-type p53 OC cell line was inhibited by increased cFLIP expression, thus preventing procaspase-8 recruitment and activation at the DISC level (185). Despite the expression of both Fas and sFas, primary OC cultures are resistant to Fas-mediated apoptosis (186).

Previous studies of OC cell lines focused on the enhancement of TRAIL-mediated apoptosis by chemotherapeutic drugs (175,181,187-190). Results from these studies demonstrated that sensitivity to TRAIL-induced apoptosis was enhanced in combination with chemotherapeutic agents in OC cell lines with variable sensitivity to TRAIL-induced cytotoxicity. Possible mechanisms for this potent apoptotic outcome of combined TRAIL-therapeutic drugs are related to increased caspase activation (175,189), upregulation of TRAIL receptors (187,190), induced expression of pro-apoptotic proteins and downregulation of anti-apoptotic proteins (190).

As a supplement to the therapeutic potential of TRAIL-based therapy in OC, substantial research has focused on the prognostic implications of DR expression and TRAIL sensitivity.

DRs and their ligands have been shown to be differently expressed in OC and interpretation of their clinical role is controversial (180,191-194). Horak et al. reported that neither DR4 and DR5 nor TRAIL expression levels were significant predictors of survival (180), whereas Duiker et al. and Lancaster et al. found that high expression of TRAIL was associated with better patient survival (191,193), with no such role for DR4 and DR5 (191). Yet another investigator has shown that high expression of DR5 was associated with a poor prognosis in patients with invasive tumors and patients with grade 3 tumors of the serous type (192). Furthermore, the combined expression of 2 proteins (TRAIL and DR5, DcR2 and Cyc, FLIP and DR5, FLIP and DcR2, DcR1 and DR5 or DR4 and FLIP) was associated with prognosis (192).

Frequent Fas expression in OC was shown in a number of studies (186,191,195,196). In contrast, in other studies widespread expression of Fas was detected in benign ovarian samples, but was almost absent in carcinoma cases (197). Positive Fas expression was associated with better progression-free and disease-specific survival in one study (191), while another study found that FasL expression in carcinoma specimens was associated with a less favorable prognosis than FasL-negative cases (196). Additionally, OC patients with a serum soluble Fas levels lower than 1.5 ng/ml had significantly higher survival rate than patients with levels above 1.5 ng/ml (198).

TNF-R1 and TNF-R2 expression has been studied in both benign and malignant ovarian cells by several investigators (199-201). TNF-R1 expression was detected in both benign and malignant lesions (199-201). The expression of TNF-R2 was reported in 51% of malignant specimens (199), but was almost absent in benign lesions (199,201). In contrast, TNF-R2 was reported to be absent in carcinoma cells (200,201), but confined to infiltrating inflammatory cells (200). Analysis of the association between TNF-R1 and TNF-R2 expression and patient outcome showed that patients with TNF-R2-positive tumors had poor survival (199).

Ascites obtained from women with advanced OC has been demonstrated to inhibit death receptor-induced apoptosis through αvβ5 integrin-mediated focal adhesion kinase and Akt activation (183,202). This observation suggests that ascites actively contributes to the progression of OC cells in effusion and that the effusion microenvironment may play an important role in regulating the resistance of OC cells to DR-mediated apoptosis. Further support of the notion that DRs are involved in OC progression is the demonstration that autocrine production of TNF-α by OC cells generates a constitutive network of other cytokines, angiogenic factors, and chemokines that may act in an autocrine and paracrine manner to promote tumor progression and development (203,204).

1.6. Caspases

1.6.1. Apoptotic function of caspases

Apoptosis, an evolutionarily conserved type of cell death, requires a specialized cellular mechanism. The main component in this pathway is a family of proteases called caspases. The study of caspases originated from the discovery that the C. elegans ced-3 gene, whose protein product is necessary for cell death in Caenorhabditis elegans, encodes a homologue of the IL-1β converting enzyme (ICE) (205). This protein was able to induce apoptosis when overexpressed in mammalian cells (205). At present, about 14 mammalian caspases have been characterized and they share a number of similar features, including amino acid sequence, structure, and substrate specificity (129,206). Caspases are synthesized as inactive proenzymes containing three domains: NH_2-terminal domain, followed by a large p20 and a small p10 subunit. These proenzymes can be cleaved to form active enzymes resulting in induction of apoptosis. All caspases, except for the serine protease granzyme B, can cleave substrates at the Asp-Xxx bond, a unique feature among mammalian proteases (129,207). Caspases contain catalytic triad residues, consisting of the active site Cys285, His237 and the

backbone carbonyl of residue 177 (129,208). They are classified into three groups based on their function: 1) Inflammatory caspases, including caspase-1, -4, -5, -11, -12, -13 and -14, which are involved in inflammatory processes rather than apoptosis. 2) Apoptotic initiator caspases, which possess long prodomains containing either a death effector domain (DED) (caspase-8 and -10) or caspase activation and recruitment domain (CARD) (caspase-2 and -9). These caspases interact with upstream adaptor molecules. 3) Apoptotic effector caspases, comprising caspase-3, -6, and -7, are identified by the presence of a short prodomain and they are processed and activated by upstream caspases, resulting in downstream execution of apoptosis by cleaving multiple cellular substrates (129,208-210). The list of proteins that are cleaved by caspases during apoptosis and/or inflammation is growing. These substrates include structural proteins, regulators of transcription/translation, kinases and signaling intermediaries (210,211).

1.6.2. Non-apoptotic role of caspases

Growing evidences for caspase activation and identification of caspase substrates in absence of apoptosis suggested a role for caspases in controlling cell proliferation, differentiation and immune functions (135,212). Proteolysis exerted by the catalytic domains and non-proteolytic function exerted by the prodomains has been indicated to be involved in the non-apoptotic functions of caspases. Furthermore, caspases may become activated independently of or without inducing the apoptotic cascade, resulting in cleavage of specific subset of substrates such as cytokines, kinases, transcription factors and polymerases to avoid cell demolition (135,212). c-FLIP, human caspase-12 and CARD-only proteins may be involved in the regulation of the non-proteolytic functions of caspases. High concentration of c-FLIP$_L$ at the level of the DISC prevents recruitment of caspase-8 to the DISC resulting in impeded caspase-8 activation that blocks cell death. Instead, c-FLIP$_L$-mediated NF-κB activation is

promoted (135,212,213). Interestingly, in addition to participation in cell death, caspase-8 and caspase-3 have also been reported to be involved in non-apoptotic functions such as embryonic development, T and B cell proliferation, macrophage differentiation and remodeling of cellular populations in response to cerebral ischemia (213-215).

1.7. c-FLIP

1.7.1. The role of c-FLIP in regulation of DR-mediated apoptosis

Apoptosis mediated by DRs is regulated by inhibitor proteins, including c-FLIP. To date, several c-FLIP splicing variants have been identified at the mRNA level. However, at the protein level only two splice variants, the long isoform c-$FLIP_L$(55kDa) and the short isoform c-FLIPs(26 kDa), were shown to demonstrate different molecular mechanisms for inhibiting the activation of pro-caspase-8 (216-218). c$FLIP_L$ is structurally similar to pro-caspase-8, containing two death effector domains (DED) at the N-terminus and caspase-like domain at the C-terminus. However, this domain lacks the catalytic cysteine residues which are essential for the catalytic activity of caspase-8, and hence, it is catalytically inactive. The short isoform c-FLIPs is structurally similar to viral FLICE-inhibitory proteins (v-FLIP) (219). Structural variations in all forms of c-FLIP reflect different functional roles, pointing to modulation of caspase-8/10 activity in the physiologic regulation of normal tissue development and in pathological conditions. All isoforms of c-FLIP, when expressed at high level, have been described to bind to FADD/TRADD using their DED motif and compete with pro-caspase-8/10 for the recruitment at the DISC, resulting in prevention of the pro-caspase proteolytic cleavage and activation. This may be the common mechanism to inhibit the transduction of apoptotic signaling and promotion of cell survival (216-219) (**Figure 15**). Despite the role of c$FLIP_L$ as an inhibitor of caspase-8 activation, other studies have demonstrated that c$FLIP_L$ is also able to trigger caspase-8 activation. This double function has been found to depend on

various parameters, including the cellular context and the caspase-8 to $cFLIP_L$ ratio (212,220-222).

Elevated expression of c-FLIP has been described in a variety of human tumors, including melanoma (216, 223), colorectal carcinoma (224,225), pancreatic carcinoma (226, 227), non-Hodgkin lymphomas (228,229), breast carcinoma (230) and OC (176-180,185,231-233). Recent findings indicate that c-FLIP expression plays a key role in conferring cellular resistance to death-receptor mediated apoptosis. Several studies have described that downregulation of c-FLIP expression results in sensitizing various types of resistant cancer cells to Fas and TRAIL-induced apoptosis (177-180,183,226,227,230-234).

1.7.2. The clinical role of c-FLIP in OC

Published data regarding the clinical role of c-FLIP overexpression in human cancers has only recently begun to accumulate (225,235-238). c-FLIP expression in OC has been analyzed in only few studies and results are inconclusive with respect to its clinical role. Two studies by Horak et al. and Duiker et al. (180,191) demonstrated no association between c-FLIP expression and survival in analysis of primary OC, while Bagnoli et al. (239) reported that combined expression of p53 and c-FLIP expression was associated with unfavorable prognosis compared to lack of both with respect to both PFS and OS in primary OC. In multivariate analysis in which the prognostic role of p53 and c-FLIP was separately analyzed, the latter protein was an independent prognostic marker (239).

1.8. Phosphatidylserine

1.8.1. Phosphatidylserine (PS) in mammalian cells

Mammalian cell membranes contain several types of phospholipids which are unevenly distributed between the two leaflets of the plasma membrane. The negatively charged PS

constitutes approximately 2-10 % of the total cellular lipid. In addition to a structural function, the preservation of PS in the cytosolic leaflet of cells has an important role in cell physiology, as it facilitates binding of proteins at the inner membrane surface, serves as a cofactor for several membrane-bound enzymes (e.g., protein kinase C), participates in signaling pathways and promotes membrane-fusion during exocytosis and similar processes (240-244).

1.8.2. Cell surface exposure of PS

PS is normally localized at the cytoplasmic side of the plasma membrane. However, certain conditions may lead to exposure of PS on the outer leaflet of the plasma membrane, where it can initiate and participate in several important biological processes, such as blood coagulation, cell clearance, immune regulation and apoptosis (240-244). PS cell surface exposure has been shown in erythrocytes, activated platelets and erythroid precursors (241,245), in undifferentiated tumor cells (246,247) and in apoptotic lymphocytes (248), as well as in cancer cell lines derived from different cancer types such as malignant melanoma, prostate carcinoma, renal carcinoma, glioblastoma and rhabdomyosarcoma (249). Riedl et al. further demonstrated that PS externalization was significantly higher in these cancer cell lines compared to the corresponding non-tumorigenic control cells, and association between the levels of PS exposure and malignancy was observed in four malignant melanoma cell lines compared to melanocytes (249). Thus, PS exposure was suggested as potential marker to differentiate between malignant and non-malignant cells, as well as an informative biomarker for diagnosing disease and a target for cancer therapy (249,250). The externalization of PS in activated platelets serves as a procoagulant surface; in erythroid cells it plays an important role in cell maturation, whereas in erythrocytes and undifferentiated tumor cells, PS exposure mediates cell recognition and phagocytosis by macrophages and other cells (241, 245-248).

Furthermore, numerous reports have shown elevated expression of PS on the outer leaflet of the plasma membrane of different cell types undergoing apoptosis, including lymphocytes, thymocytes, tumor cell lines of lymphoid and neural origin, smooth muscle cells and vascular endothelial cells (248,251-254).

1.8.3. The PS-binding protein annexin V

To date, several PS-binding compounds, including proteins, peptides and small chemical entities have been described (250). Peptides and small chemical entities have low affinity for binding PS, whereas proteins demonstrate higher affinity for binding it (250). Annexins represent a large family of closely-related calcium-binding membrane proteins that are widely expressed in eukaryotes (255). Annexin V, a 35-kDa Ca^{2+}-binding protein, was first described by Reutelingsperger et al. as a vasculature-derived protein with strong anticoagulant properties that binds with high affinity to PS (256). The affinity (257,258) and specificity (259) of this binding has been previously reported. Annexin V has been intensively investigated as a molecular imaging agent to visualize PS-expressing apoptotic cells *in vitro* and *in vivo* (260,261). Fluorochrome-conjugated Annexin V in combination with a membrane impermeable DNA dye such as propidium iodide (PI) or 7-amino-actinomycin-D (7-AAD), has been used to differentiate between viable, apoptotic and secondary necrotic cells (252,253, 262-265).

2. AIMS OF THE STUDY

Despite the established role of FCM in the diagnosis of hematological neoplasms, the use of this technique in diagnosis and clinical research of non-hematological cancers in general and effusion cytology in particular still requires improvement in way of optimization and calibration. In addition, quantitative analysis of molecules involved in apoptosis and drug resistance may represent an important tool for evaluating treatment response and prognosis in advanced and/or recurrent OC.

The aims of the study were therefore:

1. To improve the ability of FCM to detect epithelial cells in effusion by focusing on issues such as instrument settings, panel selection and specimen handling/storage.

2. To quantify apoptosis and to study the relationship between molecular markers of proliferation, survival and apoptosis in OC effusion specimens.

3. To study the association between proteins which mediate apoptosis or cell survival and clinicopathologic parameters, including chemotherapy response and survival in OC.

4. To compare the expression patterns of the above molecules in OC, breast carcinoma and MM effusions.

3. MATERIALS AND METHODS

3.1. Cell lines

Nine different cell lines, detailed in **Table 3**, were used in the present thesis as control cells. All cell lines were in-house lines originally purchased from ATCC, with the exception of OVCAR-8 cells, which were a gift from Professor Reuven Reich at the Hebrew University, Jerusalem, Israel.

3.1.1. Preparation of cell lines

All cell lines were propagated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with fetal bovine serum to a final concentration of 10%. Depending on the analysis, cells were both harvested by scraping or trypsination and analyzed fresh or after freezing in -70°C in RPMI 1640 medium supplemented with 50% FBS and 20% dimethylsulfoxide (DMSO).

Table 3. Cell lines used in the thesis

Cell lines	Origin
MDA-MB-231	Breast adenocarcinoma
SK-BR-3	Breast adenocarcinoma
T47-D	Breast adenocarcinoma
OVCAR-8	Ovarian adenocarcinoma
SKOV-3	Ovarian adenocarcinoma
OVCAR-3	Ovarian adenocarcinoma
NCI-H520	Squamous cell carcinoma of the lung
HS-Sultan	Burkitt lymphoma cells
MSTO 211H	Mesothelioma

3.2. Peripheral blood leukocytes (PBL)

Peripheral blood leukocytes (PBL) from healthy donors were additionally analyzed. Briefly, after centrifugation in lymphoprep (AXIS-SHIELD, Oslo, Norway), mononuclear cells were separated from at the sample/medium interface and the harvest fraction was washed twice by addition of 15 ml 0.9% NaCl and by centrifugation at 1000 rpm for 10 minutes. The pellet was suspended in RPMI 1640 medium with 50% FBS. Cells were counted and divided equally into each cryo-tube (Greiner bio-one). Prior to freezing, an equal volume of 20% DMSO was added and the cells were frozen at -70° C.

3.3. Clinical material

The studied specimens in this project consisted of malignant (OC, MM and breast carcinoma) and reactive fresh non-fixed peritoneal and pleural effusions submitted for routine diagnostic purposes to the Department of Pathology, Norwegian Radium Hospital, Oslo University Hospital, during 1999-2007. The material included a limited number of primary peritoneal serous carcinoma and tubal serous carcinoma specimens. As these have closely-linked histogenesis and phenotype to that of their ovarian counterparts, all are referred to as OC in paper II, IV and in this thesis.

In paper I, antigenic immunoreactivity of fresh and frozen specimens was analyzed in 10 fresh non-fixed effusions consisting of 8 AC, 1 MM and 1 one reactive specimen. In addition, 20 effusion specimens were compared using both FCM and ICC.

The material studied using FCM in paper II consisted of 95 OC effusions (80 peritoneal and 15 pleural). These were obtained from 77 patients treated at the Section for Gynecologic Oncology, Division of Obstetrics and Gynecology, Norwegian Radium Hospital, Oslo University Hospital, of whom 12 had more than 1 effusion. Additionally, suspensions from 20

non-matched primary carcinomas were analyzed following a request by one of the Reviewers to study primary carcinomas from patients who did not have effusions at diagnosis.

In paper III, 12 OC effusions were tested.

Of the 95 OC effusions in paper II, 76 specimens, consisting of 63 peritoneal and 13 pleural effusions, were analyzed for annexin V expression in paper IV.

The material studied in paper V consisted of 27 cytological specimens, including 22 effusions and 5 fine needle aspirates. Twenty specimens were malignant, including tumors of different origin, and 7 were reactive.

In paper VI, a total of 69 effusions, including 61 peritoneal and 8 pleural, were studied for c-FLIP expression. However, only a limited number of these effusions (21 to 28) were analyzed for DR and cFLIP expression in papers II and V, respectively.

3.3.1. Preparation of effusion samples

In order to avoid any bias that may be caused by different handling, and to secure minimal cell death, effusions were processed immediately after tapping with centrifugation for 10 minutes at 2000 rpm. From the resulting pellet, two Diff-Quik®-stained and two Papanicolaou-stained smears were prepared and evaluated for adequacy by an experienced cytopathologist (Prof. Ben Davidson). The specimens were considered adequate when a distinct tumor cell population was observed and cell degeneration was absent. Based on specimen volume and the number of cancer cells, the remaining material was divided for freezing in -70°C in RPMI 1640 medium with 50% FBS and 20% DMSO at ratio 1:1 and for cell block preparation using the thrombin clot method (82). Cell blocks were fixed in 4% buffered formalin overnight, as for surgical specimens, in order to avoid any methodological bias regarding sample handling in comparison of effusions with solid tumors.

3.3.2. Pathological diagnosis

An experienced pathologist evaluated and diagnosed all specimens and new cancer diagnoses on either cytology or histology samples were confirmed by a second senior pathologist. All cases, including smears and hematoxylin and eosin-stained sections from all effusions and slides from solid tumors were reviewed by Prof. Davidson.

3.3.3. Clinical data

Relevant clinical data, including patient age, FIGO stage, residual disease volume, dates of chemotherapy administration and clinical response following each chemotherapy course, as well as PFS and OS data, were obtained from the Section for Gynecologic Oncology, Division of Obstetrics and Gynecology, Norwegian Radium Hospital, Oslo University Hospital.

3.3.4. Ethics

This project was approved by the Regional Committee for Medical Research Ethics Norway (S-04300). Norwegian pertinent laws and rules, including Biobank law and Data Inspectorate Regulations, were strictly followed.

3.4. FCM immunophenotyping of cells in effusions

FCM immunophenotyping is rapid, reproducible and sensitive, and is a reliable method for detecting cellular (cytoplasmic, nuclear and surface) antigens. Multi-color FCM provides the opportunity to evaluate multiple antigens simultaneously, making it possible to characterize various cell populations in a more precise manner (84). Cytological specimens such as effusions are suitable for FCM analysis, due to the presence of viable cells in suspension. In paper I, we optimized the conditions for FCM immunophenotyping of effusion specimens

applying four-color analysis. The results from paper I provided the basis for FCM analysis of DRs and Annexin V in paper II and IV, respectively, and FCM constitutes the main technique applied in this thesis. A panel of antibodies that allows for rapid and effective differentiation between cells of epithelial, mesothelial and hematological origin has been established (84) and the basis for reliable detection of cells in effusions has been shown in previous studies (116, 266). The Ber-EP4 antibody was most frequently and widely immunoreative in AC cells (267), and was thus used in the antibody panel in all papers in the present thesis, in combination with the leukocyte marker CD45, in order to guarantee that only AC cells were analyzed for target protein expression. In addition, an EpCAM antibody, directed against the same cell surface adhesion molecule as Ber-EP4, was used as control for the Ber-EP4 reaction in apoptotic cells in papers II, V and VI. EpCAM stains epithelial cells and some mesothelial cells and is overexpressed in a variety of carcinomas (268). Characterization of MM was performed applying an anti-EMA antibody instead of Ber-EP4.

3.4.1. Control of instrument performance

FCM was undertaken using the FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a 15 mW Argon-ion laser (488 nm) and 12 mW red diode laser (635 nm). FITC (FL1, BP 530/30 nm), PE (FL2, BP 585/42 nm), PerCP (FL3, LP 670 nm) and APC (FL4, BP 661/16) measurements were collected in the logarithmic mode. Control of instrument performance and time delay calibration were performed using FACSComp software version 4.1, Calibrite™ 3 beads and Calibrite™ APC beads (all from BD Biosciences).

3.4.2. Evaluation of FCM immunophenotyping

Evaluation and scoring of FCM immunophenotyping was undertaken using CellQuest Software (BD Biosciences) in papers I and II, and FlowJo analysis software (Tree Star Inc.,

Ashland, OR) in papers III-VI. The percentage of tumor cells expressing the relevant markers was scored. Expression in <1% of cells was scored as negative.

3.5. Detection of apoptosis

Several methods have been described for detection of cell death-related parameters, including membrane alterations, mitochondrial changes, caspase activity and DNA fragmentation (269-273). Transmission electron microscopy (TEM), considered as the gold-standard test, has been used to identify the ultrastructural morphological changes in cells undergoing apoptosis (271-273). However, TEM has limitations as an apoptotic detection method, including high cost and being a time-consuming procedure, making screening of large specimens difficult. In addition, apoptotic cells detected by TEM are at the late state of apoptosis and often are deficient in the structures and antigens that would identify the cell type (271-273). For any given method, limitations regarding sensitivity (positive identification of apoptotic cells) and specificity (detection of apoptotic vs. necrotic cells) in detecting apoptotic cells need to be considered, and recognition of apoptosis is thus more reliable when more than one viability assay is performed (269,270,274). Apoptosis is an event of different induction and execution kinetics. Thus, detection of cells undergoing apoptosis generally relies on a specific marker that is expressed in variable time intervals. Awareness of the time-point in which specific markers are being detected is therefore important for the rational application of methodology (269,275).

Multicolor FCM provides the opportunity to evaluate multiple antigens simultaneously, making it possible to characterize and quantify various apoptosis features in a more reliable way (262,263, 276,277). An assay for studying apoptosis in effusion specimens, detecting

cleaved caspase expression and DNA fragmentation by the TUNEL reaction, was established in paper III. This assay was then used in paper V.

Alterations in lipid composition of the cell membrane have been reported as an early sign of apoptosis (251-253,269,273). PS exposure on the outer leaflet of the cell membrane can be detected by FCM using the fluorochrome-conjugated anticoagulant protein Annexin V (257,261,262). Although this method is quite sensitive, distinction between live, apoptotic, and late apoptotic/necrotic cells is not possible unless a plasma membrane permeability marker such as PI or 7-AAD is used in conjunction with Annexin V (261-263). Caution should be considered in interpretation of results from Annexin V assay after mechanical or enzymatic detachment of adherent cells from culture flasks, as these have been reported to increase exposure of PS, leading to experimental bias (262,278).

A hallmark of apoptosis is the activation of caspases (207,208). Under normal physiological settings, caspases are constitutively present in the cytoplasm as zymogens with low intrinsic activity, which become activated upon cleavage. The expression of cleaved caspase-3 and -8 were studied in papers III and V using specific antibodies that recognize epitopes exposed upon caspase cleavage. Other approaches were developed to detect intracellular caspase activation using fluorochrome-labeled inhibitors of caspases (FLICA) or specific fluorogenic caspase substrates (269,273). However, FLICA seems to bind additionally to targets other than activated caspases (279).

DNA fragmentation cause by endonucleases in cells undergoing apoptosis generates a multitude of DNA double-strand breaks (DSB) (280,281) and can be detected by the TUNEL reaction assay (269,273). In this method, exogenous terminal deoxynucleotidyl transferase adds fluorochrome-labeled triphosphodeoxynucleotides such as dUTP to the 3’-hydroxyl ends

of double-strand DNA, which allows for detection by image cytometry or FCM (269,281). Significant DNA fragmentation is suggested to be a specific marker of apoptosis and the number of DSBs in apoptotic cells is usually much higher than in necrotic cells, ensuring their discrimination by using the TUNEL assay (282). However, it has been shown that TUNEL is not always able to distinguish between different forms of cell death (283,284). In addition, there are cases where apoptotic or apoptotic-like cell death proceeds without extensive DNA degradation, leading to inadequate identification of apoptotic cells by TUNEL (269,274). Alternatively, identification of DNA degradation by extracting the DNA can be performed using agarose gel electrophoresis. Multiple DNA fragments 180-200 bp in length resulting from apoptosis can be visualized as a DNA ladder (285,286). As to TUNEL, this assay is also not capable of detecting apoptosis in situations where apoptosis-induced DNA degradation occurs in the absence of internucleosomal DNA fragmentation (287,288).

Several mechanisms have been described explaining mitochondrial function in apoptosis, including release of apoptogenic proteins into the cytosol upon mitochondrial outer membrane permeabilization (MOMP). The mitochondrial changes result in a release of cytochrome c and an array of cell death-modulating small proteins such as AIF, EndoG, Omi/HtrA2 and Smac/DIABLO, normally enclosed in the intermembrane space of the organelle (289). Mitochrondrial transmembrane potential loss, which is an early event in apoptotic cells, can be examined by cytometric assays using membrane-permeable lipophilic cationic fluorochromes such as rhodmaine 123, 3,3-dihexiloxa-dicarbocyanine ($DiOC_6(3)$) or 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (269,271,290). The probes are taken up by live cells, accumulate in the mitochondria, and efflux into the cytosol of apoptotic cells, leading to changes in fluorescence intensity. There are emerging data suggesting that, depending on the model of apoptosis, the collapse of

mitochondrial transmembrane potential may not be a universal requirement for apoptosis (291,292).

3.6. Immunohistochemistry (IHC)

The technique of IHC allows for the visualization of epitopes in situ in histological tissue sections or cytological smears. A series of innovations in the assays and reagents and the introduction of mechanization (293,294) have improved the ease and technical reliability of this technique leading to widespread application as a complement to morphology (104,109,295). IHC is an important tool to apply in the diagnosis of cancer of unknown origin or to determine the nature of the cells when their degree of malignancy is uncertain (294,295). However, it is now appreciated that IHC experiments may result in false-negative or false-positive staining, owing to over-fixation or poor tissue fixation with formalin, poor handling of reagents, inadequate antigen retrieval and improper antibody dilution (293-295).

In paper I, the performance of IHC in terms of sensitivity and specificity was compared to FCM. Briefly, formalin-fixed paraffin-embedded cell block sections, 4 micron-thick, were mounted onto silane-coated slides, air-dried at 60°C for 45 min and then at 37°C overnight. Slides were deparaffinized and rehydrated prior to staining. The antigen retrieval was performed by using a microwave oven. Staining was performed using the EnVision Peroxidase (DAB) kit (Dako). Appropriate positive and negative controls were used. Staining intensity and extent (percentage of stained cells) were scored. Staining intensity was scored semi-quantitatively as 0-3 corresponding to absent, weak, moderate and strong staining pattern. Staining extent was determined as the average number of cells in each X40 field throughout the specimen.

3.7. Western blotting (WB)

WB has been reported to be a potent method for the immunodetection of the presence, relative abundance, relative molecular mass and post-translational modification of proteins, following electrophoresis. The value of this technique originates from its ability to provide simultaneous resolution of multiple immunogenic antigens within a sample for detection by specific antibodies (296). However, several conditions, including lysis, antibody concentrations, the quality and volume of protein loaded, membrane background fluorescence, membrane type and porosity, washes, incubation time and exposure, may influence the final results (296).

In paper VI, WB was used to test two commercial c-FLIP antibodies in a panel of four cell lines, consisting of OC and MM. The best performing antibody was chosen for further study of effusion specimens applying FCM. In brief, cell pellets were lysed in ice-cold lysis buffer. Protein lysates (25μg per lane) were separated by SDS polyacrylamide gel electrophoresis and blotted onto Immobilon-P membranes. Membranes were blocked overnight in 5% nonfat dry milk in Tris buffered saline-Tween. Filters were incubated with anti-c-FLIP antibodies in 5% milk in TBST. The secondary HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse antibodies were applied. The enhanced luminol-based chemiluminescent plus WB detection system was used for visualization.
Antibody specificity was tested with a c-FLIP blocking peptide (2/18; Abcam) according to the manufacturer's protocol. Anti-α-tubulin mouse monoclonal antibody was served as loading control.

3.8. Statistical analysis

In all papers, with the exception of paper III, statistical analysis was performed using the SPSS-PC package (Chicago, IL). Probability of <0.05 was considered significant. In paper I, the association between FCM and ICC results was executed using Wilcoxon Signed Ranks Test, whereas, the association between the expression of the studied molecules and clinicopathologic parameters in papers II, IV, V and VI was performed using the Mann-Whitney *U* test. The same test was used in paper II for comparative analysis of receptor expression between OC versus breast carcinoma. Paired *t* test was used in papers II, IV and VI for the relationship between the expressions of the studied molecules.

Survival analysis was performed by using the Kaplan-Meier method and groups were compared with the log-rank test. Multivariate survival analysis was executed by using Cox proportional hazard model.

4. SUMMARY OF PAPERS

Paper I

Flow cytometric immunophenotyping of epithelial cancer cells in effusions – Technical considerations and pitfalls

This study focused on optimization of the technique for effusions using a 4-color analysis. FCM analysis was performed using antibodies against epithelial and mesothelial markers (Ber-EP4 and EMA), CD138 and integrin subunits. The titration of antibodies revealed the importance of this procedure prior to use of antibodies for immunophenotyping, since applying erroneous antibody dilution may lead to higher background staining and to either over- or under-compensation of the fluorescence signals. FCM of frozen vs. fresh specimens and the performance of FCM compared to immunhistochemistry were evaluated. FCM optimization was achieved and applied to clinical specimens, with resulting detection of epithelial markers and adhesion molecules on cancer cells. Frozen clinical specimens and cell lines showed reduced CD138 expression compared to fresh specimens, with conservation of the remaining epitopes. FCM generally showed comparable performance to immunhistochemistry.

Paper II

Death receptor expression is associated with poor response to chemotherapy and shorter survival in metastatic ovarian carcinoma

In this study, we analyzed death receptor expression in malignant effusions (95 OC and 9 breast carcinoma effusions). Viable tumor cells were analyzed for DR4, DR5, Fas, TNFR1 and TNFR2 expression using FCM. In addition, twenty primary ovarian carcinomas were studied. Results were analyzed for association with clinicopathologic parameters, chemotherapy response and survival. DR4, DR5 and Fas were expressed by the majority of

specimens, with less frequent expression of TNFR1 and TNFR2. OC in effusion had significantly higher Fas expression than breast carcinoma specimens (p=0.047), with non-significant differences for the remaining 4 receptors. As in effusions, primary OC showed frequent expression of DR4, DR5 and Fas, with infrequent expression of TNFR1 and TNFR2. In OC, DR4 (p=0.005) and TNFR2 (p=0.041) expression was higher in FIGO stage IV compared to stage III tumors. Effusions from OC patients who responded poorly to chemotherapy administered at disease recurrence had significantly higher DR4 (p=0.006), DR5 (p=0.01) and Fas (p=0.001) expression. In univariate survival analysis for OC patients, higher DR4 expression correlated with poor progression-free (p=0.0411) and overall survival (p=0.0352). DR4 expression in both cell populations was found to be an independent predictor of overall survival (p=0.008) and progression-free survival (p=0.003) in multivariate Cox analysis.

DR expression in tumor cells in effusions was frequently observed in both OC and breast carcinoma. The association of DR expression with advance stage, poor response to chemotherapy and poor survival may suggest that cancer cells in effusion are protected from DR-induce apoptosis and these molecules are linked to aggressive clinical course in metastatic OC.

Paper III

Methods for simultaneous measurement of apoptosis and cell surface phenotype of epithelial cells in effusions by flow cytometry

The objective of this study was to establish a FCM assay for detection of epithelial cells in effusion combined with quantification of apoptosis. The assay consisted of following stages: culturing and induction of apoptosis by staurosporine in control OC cell lines (SKOV-3 and OVCAR-8); preparation of effusion samples and cell lines for staining; staining of carcinoma

cells in effusion and cell lines using cell surface markers (Ber-EP4, EpCAM and CD45) and intracellular/nuclear markers of apoptosis (cleaved caspase-3 and caspase-8, and incorporated deoxyuridine triphosphates; dUTP); and FCM analysis of cell lines and effusion specimens. Cell lines treated with staurosporine underwent apoptosis, evidenced by considerable higher expression of the apoptotic markers compared to control cell lines. Carcinoma cells in 12 effusion specimens were differentiated from leukocytes applying the epithelial markers Ber-EP4 and EpCAM and leukocyte marker CD45, whereas mesothelial cells were excluded through the absence of expression of epithelial markers. Low percentage of apoptotic carcinoma cells in effusion specimens was observed.

Paper IV

Evaluation of cell surface expression of phosphatidylserine in ovarian carcinoma effusions using the Annexin V/7-AAD assay

This study investigated Annexin V expression in 76 OC effusions using FCM. Results were analyzed for association with clinicopathologic parameters and survival. In addition, Annexin V expression was compared with the previously-studied apoptotic markers (cleaved caspase-3 and caspase-8 and dUTP incorporation). Annexin V was detected in all specimens and the expression was significant higher compared with cleaved caspases and dUTP incorporation ($p<0.001$). Annexin V expression was higher in grade 3 tumors compared to grade 1-2 tumors ($p=0.014$). In postchemotherapy specimens, higher Annexin V expression significantly correlated with shorter OS ($p=0.005$) and progression free survival ($p=0.013$). The data presented in this study document Annexin V expression in OC cells effusions. The higher Annexin-V expression compared with cleaved caspases and dUTP incorporation may suggest that PS surface exposure may be involved in cellular processes other than apoptosis.

Paper V

Measurement of apoptosis in cytological specimens by flow cytometry: comparison of Annexin V, caspase cleavage and dUTP incorporation assays

The aim of this study was to compare the performance of different assays for measuring apoptosis in cytological specimens by flow cytometry. Apoptosis was investigated in 27 (22 effusions and five fine needle aspirates) specimens consisting of 20 malignant and 7 reactive specimens. The detection of malignant cells was confirmed in all 20 specimens using Ber-EP4, EpCAM and EMA. Wide variation in the degree of apoptosis was detected in both malignant and reactive samples applying all assays. However, the percentage of Annexin V-positive cells was higher compared with those showing caspase cleavage and dUTP incorporation in most cases, irrespective of specimen type. Both malignant cells and reactive mesothelial cells in pleural effusions had significant lower expression of dUTP incorporation compared with their counterparts in peritoneal specimens ($p=0.001$). The results of this study were in concordance with our previous observation in OC effusions, that measurement of apoptosis applying the Annexin V assay gives higher expression value than those obtained using the cleavage caspase and dUTP incorporation assays, suggesting that this assay may not accurately reflect the degree of apoptosis in both reactive and malignant cells in effusions.

Paper VI

Flow cytometric measurement of cellular FLICE-inhibitory protein (c-FLIP) in ovarian carcinoma effusions

In this study, a FCM protocol for detection of c-FLIP in serous effusion specimens was established. Additionally, the clinical relevance of c-FLIP expression and the potential association with previously studied parameters such as DR, cleaved caspases and dUTP incorporation was studied. The procedure consisted of following steps: two c-FLIP antibodies

were tested in four different cell lines of OC (SKOV-3, OVCAR-3 and OVCAR-8) and MM (MSTO-211H) origin using Western blotting and the best performing antibody was used for titration of c-FLIP expression in a panel of five cell lines consisting of OC, breast carcinoma (T47-D) and MM. The concentration that provided the best discrimination between signal and noise was applied for comparison of the performance of three fixation and permeabilization protocols. The best performing protocol was used for analysis of 69 OC effusions. c-FLIP expression was analyzed for association with clinicopathological parameters and survival. Rabbit polyclonal c-FLIP by Abcam and the IntraStain kit by Dako provided the best results. c-FLIP expression was observed in tumor cells in all 69 effusions. No association was found between c-FLIP expression and clinicopathologic parameters, including chemoresponse and survival. However, an inverse correlation was found between c-FLIP levels and expression of the previously studied apoptosis marker cleaved caspase-3 ($p=0.029$). An assay for measuring c-FLIP in cytology specimens is presented. c-FLIP is frequently expressed in OC effusions, but its expression appears to be unrelated to disease aggressiveness.

5. RESULTS AND DISCUSSION

5.1. The problem

Serous effusions are a common finding in patients suffering from advanced cancer, including AC of the lung, ovary, breast, and gastrointestinal tract, MM and primary peritoneal carcinoma, for whom the odds of getting cured by conventional treatment are low (84). This highlights both the significance of strengthening early detection and the call for developing targeted therapy approaches, such as characterization of the anti-apoptotic, survival and drug resistance mechanisms of the tumor cells in both effusion and primary tumor (84,156).
As aforementioned, FCM has the potential for quantitative measurement of molecules involved in a range of biological processes such as proliferation, adhesion, apoptosis, cellular metabolism and intracellular signaling, as well as molecules related to chemotherapy response and prognosis in advanced and/or recurrent cancer. In the analysis of leukemia and lymphoma, a number of consensus proceedings have been established (2,297,298).
However, the use of this technology in diagnosis and clinical research of effusion cytology still requires improvement in way of optimization and calibration due to the different characteristics of metastatic AC cells, MM cells and lymphoid cells.

In this thesis, we optimized the technical aspects of FCM by focusing on issues such as instrument settings, antibody titration, panel selection, specimen handling/storage and fixation and permeabilization to characterize carcinoma cells in effusion. Furthermore, we studied molecules involved in cell survival and in the apoptotic process and the molecular differences between metastatic OC, breast carcinoma and MM in effusions. We additionally analyzed the anatomic site-related expression and clinical role of these proteins.

5.2. The technical aspects of FCM in analysis of effusion specimens

FCM has become an essential laboratory technique in many clinical and research institutions. In hematological malignancies it is widely used to diagnose leukemia and lymphoma (1,2, 297,298). Recently, other diagnostic and research applications of multiparameter FCM have been explored (84,120-127,299-304). FCM is able to provide statistical accuracy, reproducibility and high sensitivity and allows simultaneous measurement of several constituents on a cell-to-cell basis. The main disadvantages of FCM are the lack of morphological visualization and structural information that solid tissue samples can provide. Furthermore, the technical considerations of FCM immunopheotyping are defined by components such as 1) careful sample handling, storage and preparation; 2) quality control of all staining and instrumentation protocols; 3) antibody titration, appropriate intracellular staining reagents; which are essential for high-quality multicolor FCM analysis (297,298,305,306).

5.2.1. Sample handling, storage, and preparation

Unlike blood samples, collection of effusion specimens involved more radical procedures with more patient discomfort than venopuncture. Thus, these specimens are considered as valuable diagnostic and research material and specimen flagging criteria related to the condition or appearance of the specimen may be used prior to rejection as unsuitable material (297). In our laboratory, effusion specimens containing few malignant cells (<5%) will not be considered as suitable for FCM. Crucial for all specimens submitted to FCM analysis is the preparation of single cells in suspension. The aim is to increase the yield of cells of interest while sustaining the integrity of cellular structure and antigenicity. The dissociation or separation of cells can be performed using different methodologies, including mechanical or enzymatic dissociation (297,307). Nevertheless, it should be noted that enzymatic cell

dissociation may results in some degradation of surface proteins and glycoproteins. Thus, enzyme-free or mechanical cell dissociation is occasionally preferable in preserving the structural integrity of membrane surface proteins for FCM analysis (307). However, for effusion specimens this is a minor issue since most effusion specimens contain single cells, the exception being when effusion specimens contain cells in small clusters, in which case mechanical dissociation applying a 70μm nylon filter is performed. This method is preferable since it is easy and rapid to perform without destroying cell integrity. In addition to cancer cells, effusion specimens may contain various cell populations including leukocytes, erythrocytes and RM cells. Although multi-color FCM provides the opportunity to analyze heterogeneous cell populations, it is frequently desirable to purify cell populations prior to FCM analysis. While the removal of leukocytes by density gradient centrifugation is no longer necessary, current FCM technology requires elimination of erythrocytes using different lysis methods (308). The lysis of erythrocytes in effusion specimens in our laboratory was performed using lysis buffer containing ammonium chloride, which does not affect the staining pattern in other cells. Importantly, the loss of cells of interest during sample preparation should be minimized.

Cryopreservation of ovarian tissue, oocytes, human embryos and hematopoietic cells using well recognized technologies that allow banking and safe recovery of tissue and cells have been reported (309,310). During cryopreservation cells suffer widespread physical and biological stresses. However, its application has an important role in basic and clinical sciences (311). The upper time limit for effusion specimen preservation has not been defined, but seems to be dependent on the nature of the specimen and the conditions in which the specimen is kept. Ideally, fresh effusion specimens should be processed and stained for FCM analysis immediately after collection. However, due to a busy hospital setting,

cryopreservation of effusion specimens provides practical advantages in terms of operator-dependent variability and use of the same antibody batch for analysis. In addition, valuable material may be aliquoted and is thereby available for further research. Selection of the appropriate procedures for sample handling, storage, and preparation depend on the cell parameters being analyzed (e.g., protein, DNA, or RNA) and the localization of the markers (e.g., cell surface, cytoplasm, or nucleus) (306). Choosing the appropriate procedure is essential for high-quality FCM analysis. The main drawbacks of cryopreservation are that they may result in cell loss and damage of epitopes leading to reduction and sometimes abolishment of reactivity for many antigens (306). Several studies have investigated the impact of cryopreservation on the cellular phenotype, survival, proliferation and differentiation on both hematological and nonhematological cell populations (312-318). However, the reported data are inconsistent. Some studies have suggested that cryopreservation of cells may effect the biological function of cells. One study showed that telomere shortening and cellular senescence in cryopreserved cells may be the consequence of the increase in single-strand breaks in telomeric DNA (314). Two other studies demonstrated that cryopreservation induces profound decrease of CD62L expression on the surface of CD34+ cells and of CD4+ and CD8+ T lymphocytes (312,313). Terry et al. demonstrated that the process of cryopreservation leads to decreased attachment efficiency of human hepatocytes, due to the significant downregulation of cell adhesion molecules including integrins, cadherins, catenins, and matrix mellaproteinases in the cultured cryopreserved hepatocytes compared to fresh hepatocytes (315).

Only one study has assessed the effect of cryopreservation on the cellular phenotype in cytological specimens (316). This study evaluated a method for long-term storage of cytological specimens at -70°C and found no significant differences in preservation, cellularity and staining intensity with PAP or Diff-Quik stains between fresh and frozen

specimens, with the exception of normal and malignant lymphocytes showing poorer preservation in frozen state. The reactivity for mucin and immunochemical stains was identical in fresh and frozen preparations, with the exception of β-glucuronidase and CEA staining, the former being negative in frozen specimens and the latter absent in fresh samples (316). In another study, the cell suspension from fresh tissue specimens was divided in four aliquots and FCM analysis was performed on fresh cells, cells in 50% methanol at 4°C, cells in buffer after formalin fixation at 4°C and cells cryopreserved at -70°C. The authors demonstrated that cryopreservation was the best method for preservation of oncoproteins and that there was a good correlation between oncoprotein expression and the quality of the DNA histograms (300). Additional studies have shown that cryopreservation of human adipose-derived stem cells under standard 10% DMSO procedure has no impact on the phenotype, proliferation or osteogenic differentiation of the cells (317,318).

The protocol for cryopreservation of cell lines and clinical specimens applied in paper I resulted in similar expression of the surface markers Ber-EP4, EMA, CD15 and α6, αV, β1(C29) integrin subunits in fresh and frozen conditions for both cell lines and clinical specimens. The exception was CD138 (syndecan-1), which was lost or reduced in frozen MDA-MB-231, SK-BR-3, T47-D, NCI-H520 cells and in clinical specimens. Tests in our laboratory have shown that cryopreservation of cells in liquid nitrogen resulted in better CD138 antigen preservation, with no significant differences observed regarding the other markers. Taken together, these results highlight the importance of correct handling and storage of specimens prior to FCM analysis.

5.2.2. Optimization of the instrument settings

To guarantee adequate performance of the flow cytometer for any given application, the instrument and instrument settings must be properly set up and the measurement of

fluorescence intensity must be validated (297). This can be accomplished by using a range of commercially available standards counting microbeads and cellular control materials, which has been pivotal in reaching this purpose (319,320). Two sets of protocols can be carried out in order to ensure optimal instrument performance. The first set of protocols including an examination of the efficiency and performance of the lasers, optical filters, amplifiers and PMTs is performed by service personnel, whereas the second set of protocols consists of frequent monitoring of instrument set-up and performance by the operators to identify problems and variations (297,321). Optimal instrument settings entails that all cellular populations are observable on each FL or light scatter channel. This procedure is performed by applying a representative stained cell line or cell specimen (297). Correction or compensation of emission spectra overlap is of the utmost importance for proper data analysis. It is vital to understand the effects of different types of errors regarding compensation in order to recognize the inherent limitations of the data, as well as how these limitations might be dealt with by changes in experiment design (322). Several approaches for multicolor compensation based on hematopoietic cell populations have been described (16,17,323-325). In paper I, however, due to the different characteristics of epithelial cells regarding cell size, cell complexity and autofluorescence background compared to hematopoietic cell populations, the PMTs and instrument settings were optimized using a single-stained mixture of T47-D/PBL for detection of the cells of epithelial origin. The subtraction of unwanted contribution of fluorescent light from a dye with an overlapping emission spectrum was performed using electronic hardware subtraction. The compensation was carried out using fluorochrome (FITC, PE, PerCP and APC)-labeled monoclonal antibodies and the spectral characteristics of these fluorochromes are quite stable and alike. Thus, the same voltages for PMTs were applied throughout the thesis.

Most of the FCM instruments provide pairwise compensations. The flow cytometrer applied in the thesis allows pairwise compensation and although successful for the compensation settings in paper I, this strategy becomes increasingly complicated as the number fluorochromes increases or tandem dyes are used and may lead to artifactual data and misinterpretation (16,323,326,327). These problems can partly be solved by software compensation, when the data are collected uncompensated, providing the flexibility of setting proper compensation every time for every specimen (326). However, software compensation strategies also encounter problems as discussed by Roederer (322) and Stewart et al. (326). In recent years, remarkable advances in FCM technology regarding hardware, software tools, instrument calibration and quality control kits made it more convenient to perform instrument calibration and to measure as many as 20 independent parameters on each cell (328).

5.2.3. Antibody titration and cell fixation and permeabilization

The antibody titration assay is crucial prior to immunophenotyping, and several reports addressing this concern and providing guidelines have been published (298,329,330). When applying an antibody, titration assay is essential to optimize the amount and concentration of the antibody in order to reduce nonspecific antibody binding. The purpose of an antibody titration assay is to determine an appropriate antibody concentration resulting in the highest signal of the positive cell population and the lowest signal of the negative cell population (298,329,330). Sensitivity of a reagent determined by factors such as the cell´s autofluorescence in the region of the spectrum, the performance of the antibody conjugate and the presence of other antibody conjugates attached to the same cell are important to consider when developing multicolor antibody panels (331). The chosen panel(s) of antibody conjugates must be validated. First, the spectral overlaps of the chosen of antibody conjugates should be satisfactorily controlled. A preferable antibody conjugate to detect antigens

expressed at low intensity is PE due to its high quantum yield and lack of interference by cellular autofluoresence. Second, steric hindrance should be minimized or eliminated between the antibodies in each cocktail. Thus, the antibodies in combination should provide the same intensity as that obtained in single-color assay. Last, the volume of the antibody used for staining must be validated for the staining procedure and type of specimens used.

An increase of fluorescence intensity in negative cells, which are not supposed to express the antigen of interest, is generally due to nonspecific antibody binding. When negative cells show an increase of nonspecific antibody binding, it is likely that it also occurs additionally to the expected specific binding on positive cells. From the results in paper I, using an excess amount of an antibody may lead to increase in background staining or nonspecific antibody binding resulting in imprecise compensation and reduce sensitivity in a multicolor analysis (331,332). In paper VI, we observed that the antibody from Santa Cruz Biotechnology generated the highest S/N ratio at the concentration of 4µg without reaching an optimal antibody concentration plateau in all cell lines but one (T47-D) (data not shown). The optimal concentration of this antibody may be even higher than 4µg. The antibody from Abcam demonstrated an optimal antibody concentration plateaus at 2.5µg for all cell lines. Exclusion of an antibody from the study panel should be considered when the concentrations of the antibody are too high without reaching an optimal antibody concentration plateau, possible due to low affinity and specificity of the antibody.

In paper I and VI, titration results revealed that some vendors provide antibodies at higher concentrations than stated, whereas others provide antibodies below titer. In addition, in paper VI, validation of specificity of a marker to the condition of the material prior to immunophenotyping was performed by Western blotting.

The protocols for intracellular antigen detection have their own limitations and challenges as compared with those used for surface antigen detection. Procedures for staining of surface antigens have been successfully standardized. However, it is evident that no single method is proper for staining of all intracellular antigens. Several aspects including knowledge of antigen location, selection of fixative and permeabilization reagents, antibody specificity, fluorochrome selection and use of adequate controls need to be considered when approaching the development of an intracellular staining method (333). At present, several protocols for detection of intracellular/nuclear antigens have been described (333-340). Intracellular labeling of cells is accomplished by fixation and permeabilization to allow passage of the antibodies into the cytoplasm and nucleus without destroying the structural integrity of the cells. Awareness of the effects of various reagents used for fixation and permeabilization, as well as conditions that may be harmful for one parameter while being optimal for others is crucial (297). Fixatives can be divided on the basis of their cross-linking or coagulant characteristics. Cross-linking fixatives such as paraformaldehyde have been widely used in the targeting of intracellular antigens and have been reported to reliably anchor and stabilize most antigens, hence preventing loss of antigens after addition of permeabilzation agents and therefore making it generally the fixative of choice (333). The selection of the fluorochomes and the antibodies is very important. For membranous labeling of cells, a fluorochrome must not be altered by subsequent fixation and permeabilization. For intracellular staining, the size of a fluorochrome is important to ensure proper penetration of the conjugated antibody through the cell membrane to the target antigen. In paper III, we observed degradation of the fluorochrome PerCP but neither PE nor APC conjugated to CD45, a marker already bound to the cell surface, by ethanol fixation, resulting in lost detection. The fluorochrome PerCP, which is a natural light-harvesting carotenoid-protein complex, is probably more sensitive to ethanol denaturation than PE or APC. In paper VI, the low S/N ratio using Fix & Perm kit

compared with IntraStain kit and formaldehyde/Triton X-100 fixation and permeabilzation method may be explained by decreased of fluorescence signal of the investigated marker or increased of baseline auto-fluorescence. Furthermore, the formaldehyde/Triton X-100 fixation and permeabilzation method induced significant modification on light scatter properties resulting in formation of cell debris. On the contrary, the IntraStain kit induced only minor decreased in both FSC and SSC without causing loss of resolution or problems related to analysis. The result using the formaldehyde/Triton X-100 fixation and permeabilzation method may be a consequence of using a higher concentration of paraformaldehyde than 4%. It has been shown that using too concentrated cross-linking agent, extensive cell clumping may occur (334). In addition, the use of Triton X-100 as a permeabilizing agent has been reported to induce dramatic alteration in FSC/SSC parameters, making it difficult to distinguish lymphocytes from monocytes, as well as dead cells from live cells (337). These changes on light scatter properties as well as the increase of baseline auto-fluorescence have been reported by other investigators (333-340). The cause of autofluorescence using paraformaldehyde as a fixative may be that this agent reacts with a variety of free amine groups producing fluorescent product (340). Washing out the fixative and storing the specimens in buffer may reduce the increase in autofluorescence (336). In paper III and VI, washing out the fixative reduced but did not remove the increase in autofluorescence.

5.3. The biological role and clinical relevance of DR expression in OC effusions

Activation of DRs has been shown to promote both cell death and cell survival and proliferation (158-160,163-166,170-172).

The results in paper II showing that DRs expression in OC cells is associated with poor response to chemotherapy and poor survival may suggest that malignant ascites from OC

patients protects OC cells from DR-induced apoptosis and that these receptors may otherwise promote cell survival. This notion is supported by Lane et al. showing that some OC ascites specimens inhibit TRAIL- and FasL-induced apoptosis *in vitro* and that the prosurvival activity was dependent upon the activation of Akt (183). In another study, the same group demonstrated that malignant ascites from OC patients protects OC cells from TRAIL-induced apoptosis through Akt activation in an αvβ5 integrin-dependent pathway (202). Further evidence of the involvement of DRs in OC progression is the finding that autocrine production of TNF-α by OC cells isolated from malignant ascites generates a network of growth factors that may act in an autocrine and paracrine process to promote tumor growth (203,204). Although both Fas and sFas were highly expressed, primary OC cultures were more resistant to Fas-mediated apoptosis compared to their normal counterparts (186), and OC ascites showed the highest level of Fas compared to primary and recurrent solid specimens (341).

The addition of ascites from a cohort of 35 OC patients to the cancer ovarian cell line CaOV3 increased TRAIL IC_{50} *in vitro* and patients in the group with higher IC_{50} was associated with shorter disease-free survival (342). In other studies, DRs or ligands have been shown to be associated with outcome in OC patients. In one study, the inhibitory effect of OC ascites was associated with platinum resistance (343). In another study, the authors found that high level of DR5 was correlated with less favorable prognosis in cancer patients with invasive and grade 3 tumors (192). Tumor microenvironment-related factors, including cytokines and hypoxia, have been shown to modulate the response of cancer cells to TRAIL (344,345). Taken together, OC cell survival in the peritoneal cavity despite chemotherapy may be due to the action of anti-apoptotic factors and/or growth factors in ascites that support tumor growth and progression, hence causing tumor relapse.

5.4. DR as therapeutic targets

Various stimuli such as growth factor withdrawal, UV light, irradiation or chemicals can induce apoptosis. The latter two have been intensively used in cancer therapy (133,147). A main factor in stress-induced apoptosis through the intrinsic pathway is the activation of p53 (147). Cancer cells can obtain resistance to apoptosis by a variety of mechanisms that interfere at different levels of apoptosis signaling. The inactivation of p53 is commonly found to confer cancer cell resistance to conventional therapy (147). Hence, in order to bypass the need for p53 intact signaling, extensive efforts have been focused on the development of new therapeutic strategies targeting DRs for cancer therapy (346,347). The treatment strategy applying systemic administration of TNF and FasL has been evaluated, but its use has been hampered by undesirable toxic effects to normal tissues (348,349). TNF-α has been shown to be an efficient anticancer agent in several *in vitro* and *in vivo* preclinical studies. However, the use of systemic TNF-α has been limited due to the toxic side effects and lack of efficacy at maximum tolerated dose. Nevertheless, the combined use of TNF- α and chemotherapy in the isolated limb perfusion setting appears to be of clinical value due to its direct anti-proliferative effect of TNF-α and the capacity to enhance drug diffusion into tumor tissue (350).

Preclinical studies investigating the therapeutic role of recombinant TRAIL have shown its capacity to induce apoptosis in various tumor cell lines and xenografts, while lacking toxic effects on most normal cells (351,352). Lately, recombinant TRAIL has been used in clinical trials for the treatment of a variety of malignancies (353,354). While, results from phase I and II studies may suggest tolerated toxicity, the therapeutic efficacy has been limited (352). In addition to most of the current clinically used chemotherapy agents such as cisplatin, doxorubicin, 5-fluorouracil and camptothecin, various TRAIL and chemotherapy

combinations have been demonstrated to synergistically enhance TRAIL-mediated apoptosis (175,181,187-190,351,352). The possible values of TRAIL-induced apoptosis as an anticancer modality have been further indicated by its capacity to improve the efficiency of radiotherapy (351,352). Hence, TRAIL-driven DR activation may have great potential as a modality for cancer treatment.

Monoclonal antibody technology has been shown to be a potent therapeutic agent for cancer treatment. Preclinical studies have demonstrated the possible application of agonistic monoclonal antibodies against DR4 and DR5 for cancer therapy. The use of agonistic antibodies may have greater therapeutic potential than TRAIL due to specific targeting of DRs without decoy receptor binding. In addition, TRAIL has a much shorter plasma half-life *in vivo* compared with monoclonal antibodies (351,352). The results of monoclonal antibodies against DR4 (Mapatumumab, HGS-ETR-1, TRM-1) and DR5 (Tigatuzumab, CS-1008, Lexatumumab, HGS-ETR-2, Conatumumab, AMG 655, 4H6, Apomab) currently in phase I and II clinical trials indicate some anti-tumor activity, have a good safety profile and appear to be well-tolerated (161,351,352).

Although previously published data and the results from paper II may suggest that TRAIL sensitivity is altered in some OC (180,193,194), recent data have shown that agonist monoclonal antibody (TRA-8) against DR5 induces dose-dependent cytotoxicity in most of ovarian tumors in an *ex vivo* model. Furthermore, the combined use of TRA-8 and chemotherapeutic drugs increased the cytotoxicity (355,356).

5.5. Detection of apoptosis in malignant and reactive cytological specimens

In paper III, we have developed a FCM assay quantifying cleaved caspase-3, 8 and DNA fragmentation in apoptotic OC cells in effusion specimens (discussed in section 3.5). This assay was applied in paper V.

Based on the level of cleaved caspase-3 and -8 and dUTP incorporation, the results in paper V demonstrated that both cancer cells and RM cells in effusions undergo little apoptosis. These results are in agreement with our previous findings in the analysis of OC effusion specimens, in which detection of cleaved caspase-3 and -8 and dUTP incorporation was less than 10% of OC cells in the majority of specimens (357). Further support of the results is the observation that p85-PARP, the product of PARP cleavage by active caspase-3, is expressed in only 0-5% of OC cells in most of the cases (358). No significant differences in the levels of activated caspases and dUTP incorporation were observed in malignant and reactive specimens. This may indicate that RM cells in effusions are sentinel cells that can sense and respond to signals within their microenvironment in order to adapt, proliferate and undergo changes such as epithelial-mesenchymal transition, which is a unique feature of cancer cells (359).

5.6. Annexin V expression is not a sign of apoptosis, and is associated with poor differentiation and prognosis

Certain conditions such as blood coagulation, cell clearance, immune regulation and apoptosis (240-244) may induce translocation of PS on the cell surface and can be detected by its binding to the protein annexin V (257,258). In paper IV and V, we analyzed PS expression in OC effusion specimens and malignant and reactive cytological specimens, respectively. In both studies, we found significantly higher annexin V expression compared to other apoptosis parameters such as cleavage caspases and dUTP incorporation in most of cases, regardless of specimen type. These findings are in agreement with other studies showing higher fraction of

hematopoietic cancer cell lines expressing annexin V compared with those positive for the TUNEL assay, the Apo2.7 assay (detecting the exposure of the mitochondrial membrane protein 7A6 antigen on the cell surface) or the DNA fragmentation assay (360,361).

Elevated PS externalization on the cell surface is not inevitably activated and controlled by the apoptotic regulatory machinery (362,363). The authors further demonstrated that PS exposure is inducible, reversible, and independent of cytochrome c release, caspase activation and DNA fragmentation (363). Furthermore, PS exposure can be observed in nonapoptotic cells, including erythrocytes, activated platelets, erythroid precursors, and undifferentiated tumor cells as well as in cancer cell lines derived from different cancer types such as malignant melanoma, prostate carcinoma, renal carcinoma, glioblastoma and rhabdomyosarcoma (241,245-247,249). PS has additionally been reported to be a constituent in membrane vesicles shedding from practically all cell types and are involved in physiological and pathological processes including tumorigenesis (364). Other investigators have demonstrated that PS exposure can occur in a reversible manner under conditions of cell stress that do not necessarily obligate a cell to undergo programmed cell death (365-367). These studies revealed that PS externalization could be detected in cells with no other morphological features of apoptosis. In addition, the levels of PS exposure may be reversible on withdrawal of apoptotic stimulus (365-367).

In paper IV, high annexin V expression was correlated with poor differentiation and prognosis in postchemotherapy effusion specimens. We do not attribute this finding to mechanical PS exposure, since cells in effusions do not undergo this procedure. Furthermore, our clinical findings, together with the abovementioned studies, may suggest that PS exposure on cancer cells is not a general feature of apoptosis (249,367), but may drive the course of tumor

progression by stimulating a number of anti-inflammatory responses, inducing a state of immunosuppression and the release of transforming growth factor (TGF)-β1, an essential mediator of the process of malignant progression (241,247,364,368). Cancer cells have been shown to be able to produce non-physiological levels of TGF-β1 in an autocrine and a paracrine manner, effecting the tumor environment, contributing to tumor progression and metastasis (369). Lima et al. demonstrated elevated production of PS-containing tumor-derived microvesicles *in vitro* by the highly metastatic melanoma cell line B16F10, and these vesicles increased TGF-β1 production by cultured macrophages and, *in vivo*, enhanced the metastatic potential of B16F10 cells in mice (370). Additional evidence is the observation that formation of membrane vesicles containing PS is associated with the tumor phenotype, as evidenced by the higher amount of microvesicles found in the fluids of cancer patients compared with healthy persons (371,372). Further support of the notion that PS translocation to the outer leaflet of the cell membrane is not a sign of apoptosis is the low level of apoptotic cells detected by the TUNEL and cleavage caspases assays in paper V and in our previous study (357). In contrast, our previous study of the same cohort demonstrated that high cleaved caspase-3 expression was beneficial for patient survival, indicating that PS exposure may promote OC cell survival (357). Other studies on the prognostic value of PS exposure in OC specimens are not available. However, some reports have proposed PS cell surface expression as a potential diagnostic marker, as well as target for cancer therapy (249,250). Taken together, the results in paper IV and V indicate that the annexin V assay may not be a reliable method for apoptosis measurement.

5.7. c-FLIP is frequently expressed in OC effusions, but is unrelated to clinicopathological parameters and survival

The DR signaling pathway is regulated by inhibitor proteins such as c-FLIP, which is one of the key determinants of resistance to DR-mediated apoptosis (216-218).

Expression of c-FLIP was detected in OC cells in all effusion specimens analyzed in paper VI. We found no association between c-FLIP expression and clinicopathologic parameters, including chemotherapy response and survival. Search for studies regarding the clinical role of c-FLIP expression in OC effusions did not identify any previous publications. Some data are available, though, for primary OC, and the results are inconclusive with respect to its clinical relevance (180,191,239). In two reports, no association between c-FLIP expression and survival was observed (180,191), whereas in one study combined expression of p53 and c-FLIP was associated with poor PFS and OS compared to lack of expression of both markers. c-FLIP was an independent prognostic marker in multivariate analysis (239).

Elevated expression of c-FLIP has been found in a number of different cancers. Studies of cell lines have shown increased levels of c-FLIP in ovarian, colorectal, gastric, breast, pancreatic, and prostate carcinoma, as well as in melanoma and glioblastoma (373). Reports on primary tumor tissues from patients have also shown elevated levels of c-FLIP in malignant cells in colorectal carcinoma (225,238), B-cell chronic lymphocytic leukemia (228), bladder carcinoma (235), hepatocellular carcinoma (237), cervical carcinoma (374), malignant melanoma (375), Burkitt's lymphoma (376), and head and squamous cell carcinoma (377). Analysis of primary tumor cells from cancer patients also confirmed upregulation of c-FLIP in melanoma (236), gastric carcinoma (378) and Hodgkin's lymphoma (379). In most malignant tumors, the c-FLIP$_L$ isoform was shown to be overexpressed. However, there are

some reports demonstrating increased c-FLIP$_S$ expression (218,373). Overexpression of c-FLIP is implicated in TRAIL resistance and chemotherapy resistance, and studies have shown that high levels of c-FLIP are associated with unfavorable clinical outcome and may be a prognostic factor in cancer (225,229, 235,237,238,374-377). Increased of c-FLIP expression has been reported in gastric cancer and was associated with lymph node metastasis, thereby possibly contributing to tumor progression (378). c-FLIP was shown to expressed in both pancreatic intraepithelial neoplasms and pancreatic ductal adenocarcinomas, but absent in normal pancreatic ducts (380).

In paper II, DR expression in OC effusions was associated clinical parameters of aggressive disease, including unfavorable PFS and OS. Hence, c-FLIP expression was subsequently analyzed for potential association with DR expression. No association was found between c-FLIP and DR expression. The reason for the lack of association in our studies may be related to a small series of matched cases in both studies. Despite the small series, this lack of association may suggest that the existence of c-FLIP and DR in effusions is not inversely correlated in terms of either expression level or clinical impact. In addition, analysis of the association between c-FLIP expression and cleaved caspase-3 and -8 and dUTP incorporation (357) in a limited number of matched cases demonstrated inverse correlation between c-FLIP and cleaved caspase-3 expression. This may indicate that c-FLIP inhibits apoptotic signaling as measured by caspase-3 cleavage.

The above-mentioned studies demonstrate that c-FLIP is often overexpressed in cancer. Thus, c-FLIP may be a target for a therapeutic intervention.

5.8. c-FLIP as therapeutic target

There is evidence of strong association between the overexpression of c-FLIP and resistance to DR-mediated apoptosis in several human malignancies (373). Resistance was suggested to take place at the level of the DISC formation, where the elevated level of c-FLIP expression hampers procaspase-8 recruitment and activation (185,218). Overexpression of anti-apoptotic proteins in cancer cells suggests they may be targets for anti-cancer therapeutic intervention. Indication that DRs could induce resistance to apoptosis was reported (373). Furthermore, cancer cells can become resistant to chemotherapeutic agents. However, the combination of DNA-damaging agents or metabolic inhibitors such as 5-fluorouracil together with TRAIL can bypass this resistance in a variety of tumor cells (373). Therefore, sensitization of cancer cells to DR-mediated apoptosis can be accomplished by several stimuli inducing the decrease of c-FLIP expression, including antisense cDNA constructs, short interfering RNAs, proteasome inhibitors, protein or RNA synthesis inhibitors or chemotherapeutic agents (373).

In OC, c-FLIP expression inhibits the extrinsic pathway in the presence of functional p53 (185,381). Cisplatin, in particular, has been studied in OC cell lines and found to induce ubiquitination and degradation of c-FLIP by enhancing FLIP-p53-ITCH interaction in a p53Wt-dependent manner (382). The same investigators demonstrated that ubiquitination of c-$FLIP_{S/L}$ was under the control of the Akt pathway (383). Histone deacetylase (HDAC) inhibitors, which have been found to regulate c-FLIP levels, may be of potential value in cancer treatment. Alterations in histone acetylation patterns in OCs have been observed (154). Treatment of OC cell lines with HDAC inhibitors was shown to induce activation of caspase-9 and -3, leading to apoptotic cell death (384). Treatment with Trichostatin A, a HDAC inhibitor, sensitizes OC cells to TRAIL-induced apoptosis by decreasing c-$FLIP_L$ expression via inhibition of the EGFR pathway, with no impact of c-$FLIP_S$ (385).

Proteasome inhibitors have been shown to decrease proliferation and induce apoptosis in several malignancies (373). PS-341 (bortezomib) has been widely evaluated in different cell lines, with different outcome dependent on the type of cell line evaluated. c-FLIP expression in chronic lymphocytic leukemia, Burkitt lymphoma, multiple myeloma and esophageal squamous cell carcinoma cell lines was decreased after treatment with PS-341 (bortezomib) (373). Likewise, studies using MG-132 have shown a decrease in c-FLIP expression in chronic lymphocytic leukemia and Burkitt lymphoma cells. The decreased c-FLIP levels were associated with upregulation of TRAIL and its DR4 and DR5 (373). In contrast, in OC cell lines and OC cells, proteasome inhibitors were shown to exert a considerable pro-apoptotic effect and to enhance the sensitivity of cells to TRAIL-induced apoptosis, but this enhanced sensitivity was not directly correlated to c-FLIP degradation (386-388).

Inhibiting translation through RNA interference is regarded as the most specific technique of downregulating c-FLIP, and this strategy has been applied in several *in vitro* studies to sensitize cells to TRAIL- or FasL-mediated apoptosis (373). Saulle et al. demonstrated that c-FLIP knockdown of A2780 and A2780/ADR OC cell lines that express c-FLIP, using a specific anti-c-FLIP siRNA significantly reduced c-FLIP expression and clearly enhanced TRAIL-induced apoptosis (388). Despite promising results from *in vitro* studies, there are limitations for siRNA *in vivo*, and clinical trials applying siRNA to target c-FLIP are still not initiated (373).

In summary, c-FLIP is an important regulator of DR-mediated apoptosis and uncontrolled c-FLIP expression is correlated with malignancy in several organs. Thus, monitoring c-FLIP expression may be of diagnostic value, and agents that explicitly modify c-FLIP expression may be of therapeutic benefit.

5.9. The clinical role of cancer-associated molecules in pre- and post-chemotherapy effusions

In addition to comparison of primary tumors, solid metastases and malignant effusions, our research group also focuses on the expression and clinical role of cancer-associated molecules in pre- *vs.* post-chemotherapy effusions (84). The expression of cancer-associated molecules in malignant cells in pre-chemotherapy samples may characterize the real genetic profile of the tumor, since the cells have not yet been exposed to chemotherapy. In contrast, modification of cancer-associated molecules in malignant cells in post-chemotherapy effusion samples may in fact be related to genetic unsteadiness along tumor progression and chemotherapy treatment.

In this thesis, differences regarding the clinical value in pre- and post-chemotherapy effusions were only seen for Annexin V. In paper IV, when patients with pre- and post-chemotherapy effusions were analyzed separately, higher than median Annexin V expression in post-chemotherapy effusions predicted poor OS and PFS survival, with no prognostic value in patients with pre-chemotherapy effusions. In our previous study of the same cohort, cleaved caspase-3 levels in patients with post-chemotherapy effusions were correlated with improve survival (357). This observation is consistent with earlier work from our group showing better capacity to predict patient survival found on the expression levels of diverse cancer-associated molecules in post-chemotherapy compared to pre-chemotherapy effusions (reviewed in 84). The correlation between cancer-associated molecule expression in post-chemotherapy effusions, which are normally collected at disease recurrence at the end-time point of PFS, and PFS represents retrospective information with respect to disease aggressiveness. On the contrary, the correlation between cancer-associated molecule expression and response to chemotherapy administered at disease recurrence is a forward estimation of patient outcome. Both factors were evaluated in our material.

5.10. Apoptosis and cell survival of OC cells in effusions

Our results in papers II and IV-VI suggest that the aggressiveness of OC cell in effusions partly can be explained by aberrant apoptotic machinery and elevated cell proliferation. The low level of apoptosis, verification by low expression of parameters of apoptosis, including cleaved caspase-3, -8 and dUTP incorporation (357), may be a consequence of blockage of DR-induced cell death or the intrinsic apoptotic pathway. This is supported by the frequent expression of c-FLIP, an inhibitor of caspase-8 activation at the DISC level, which is inversely related to cleaved caspase-3 expression. Further support is the abundant expression of XIAP and Survivin, which are potent inhibitors of caspase activity, found in effusion specimens by our group (84,156). In another study from our group high level activation and expression of NFκB p65 in advance-stage OC was observed and nuclear expression was associated with poor PFS, supporting its role in cancer cell survival (84,156).

The findings that DR expression level is a marker of aggressive clinical course and chemoresistance may point to the fact that DR signaling is redirected from apoptosis to cell survival in OC cells. Furthermore, DR signaling does not only result in activation of effector caspases and following apoptosis, but can also stimulate non-apoptotic pathways, including activation of NFκB, PKB/Akt and MAPKs (389).

The Annexin V assay is not in concordance with cleaved caspase and dUTP incorporation assays in detecting apoptotic cells in effusions, and as apposed to clevead caspase-3, higher Annexin V level is correlated with poor PFS and OS, suggesting that PS exposure may be involved in cellular processes other than apoptosis.

5.11. Technical considerations

FCM is the main technique applied in this thesis and the attempt to improve multicolor FCM for analysis of epithelial cells in effusion was successfully accomplished. However, possible limitations and weaknesses of the methodology applied in this thesis must be acknowledged:

1. Sample handling, storage and preparation. Proper specimen handling is essential to avoid induction of artifacts. The effusion specimens in this thesis have been frozen for different periods of time, which could affect the final results regarding antigen preservation and cell viability.

2. Selection of antibodies/antibody combinations which are specific and sensitive for FCM analysis of carcinoma cells in effusions. The specificity and sensitivity of antibodies vary considerably from one manufacturer to another. We attempted to use the best antibodies available, but did not test all potential antibodies against each epitope.

3. Studying single cells in suspension is obligatory for FCM analysis. It may be difficult to apply the right cell dissociation method to increase the yield of cells of interest while sustaining the integrity of cellular structure and antigenicity.

4. Relatively small series of cases in some of the studies in this thesis may result in Type II error, e.g. in the analysis of the association between c-FLIP, DRs and caspases.

5. To make the data regarding DR and c-FLIP more robust, additional methods such as IHC and WB could have been applied.

6. CONCLUSIONS

1. The attempt to improve the ability of four-color FCM to study cancer-associated molecules and parameters related to apoptosis in effusion specimens was successfully accomplished. We have further highlighted the importance of correct validation of a new assay by focusing on parameters such as instrument settings, antibody titration, antibody panel selection, fixation and permeabilization, and specimen handling/storage.

2. High expression of DR4, DR5 and Fas in OC effusion specimens is correlated with poor response to chemotherapy at disease recurrence. High DR4 expression in OC effusions is associated with poor survival. DRs may mediate cell survival rather than apoptosis in these cells.

3. Significantly higher annexin V expression was detected in both reactive and malignant cells in effusion specimens compared to cleavage caspases and dUTP incorporation. In OC effusions, higher annexin V expression correlated with poor differentiation and poor survival in post-chemotherapy patients, whereas cleaved caspase-3 expression was associated with improve survival. These findings may suggest that the annexin V is not a specific marker for apoptosis measurement, but may have a role in cell survival rather than apoptosis.

4. High c-FLIP expression was found in OC effusions, but was unrelated to clinicopathologic parameters and survival. c-FLIP expression is inversely related to cleaved caspase-3 expression. OC cells in effusions undergo little apoptosis, which may be in part attributed to frequent expression of c-FLIP.

5. In view of the different expression patterns and clinical role of apoptosis-related proteins in OC effusions, the latter may be capable of activating alternative survival pathways, mediating the aggressive clinical behavior of this cancer. Thus, apoptosis determination of metastatic disease, including OC effusions, may have an important role in monitoring chemotherapy or targeted therapy response, stressing the need for trustworthy quantitative apoptotic assays.

7. FUTURE PERSPECTIVES

Over the last few decades, FCM has been established as an important technology in the diagnosis of hematological malignancies and has evolved from single-color analysis of surface membrane antigens to simultaneous multi-color analyses of surface membrane and intracellular/nuclear antigens in cell suspensions. The huge increase in the improvement and development of reliable electronics, lasers, fluidics and data analysis software, as well as an expanding range of flourochromes, has facilitated the expansion of new FCM applications as well as perfection of existing applications. Furthermore, FCM instruments have become more user-friendly and less expensive, with an expanding contribution to diagnostic medicine and basic biological research. Data from our previous studies and studies in this thesis demonstrate the potential of FCM in the diagnosis of effusion specimens and in studying cancer-associated molecules, including parameters related to cell proliferation, survival and apoptosis, with the aim of better understanding the biological mechanisms that maintain and allow cancer cell to survive at this anatomic site.

Technological developments in FCM will go on to give possibilities for increasingly advanced analyses of clinical specimens regarding diagnosis and classification of disease, prognostication and disease monitoring through and beyond treatment and in cancer research. Analysis using multiple parameters simultaneously may be beneficial for unraveling the mechanism of action of different cancer-associated molecules and parameters related to OC tumor biology in effusion material. Future potential applications of FCM in analysis of effusion specimens include validation of gene products obtained by high-throughput analyses, quantitative measurement of expression of molecules related to chemotherapy resistance, and phospho-FCM decoding of intracellular kinase signaling cascades applying specific antibodies which differentiate between the phosphorylated and non-phosphorylated states of

proteins as well as particle (microbead)-based FCM assay use to capture the soluble protein analyte from the supernatant of effusions.

An augmentation in the number of parameters is also related with higher demands in maintaining a quality system. It is crucial to pay close attention to the many technical aspects of FCM that must be mastered in order to produce high-quality results.

8. REFERENCES

1. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood 2008;111:3941-3967.

2. Greig B, Oldaker T, Warzynski M, et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: recommendations for training and education to perform clinical flow cytometry. Cytometry B Clin Cytom 2007;72 Suppl 1:S23-33.

3. Corver WE. Multiparameter DNA Flow Cytometry of Human Solid Tumors. Technical improvements and applications. PrintPartners Ipskamp, Enschede 2001.

4. Givan AL. Flow cytometry: an introduction. Methods Mol Biol 2004;263:1-32.

5. Ibrahim SF, van den Engh G. Flow cytometry and cell sorting. Adv Biochem Eng Biotechnol 2007;106:19-39.

6. Dean NP. Flow Cytometry instrumentation. In Robinson JP, Darzynkiewicz Z, Dean NP, Dressler GL, Rabinowitch SP, Stewart CC, Tanke JH, Wheeless LL, editors. Current protocols in cytometry, New York, John Wiley & Sons, inc., 1997;1.0.1-1.1.8.

7. Shapiro HM. Practical Flow cytometry – 4th ed. New Jersey, John Wiley & Sons, inc., 2003.

8. Krishhan VV, Khan IH, Luciw PA. Multiplexed microbead immunoassays by flow cytometry for molecular profiling: Basic concepts and proteomics applications. Crit Rev Biotechnol 2009;29:29-43.

9. Chapman GV. Instrumentation for flow cytometry. J Immunol Methods 2000;243:3-12.

10. Shapiro HM. The evolution of cytometers. Cytometry A 2004;58:13-20.

11. Jaroszeski MJ, Radcliff G. Fundamentals of flow cytometry. Mol Biotechnol 1999;11:37-53.

12. Henderson LO, Marti GE, Gaigalas A, et al. Terminology and nomenclature for standardization in quantitative fluorescence cytometry. Cytometry 1998;33:97-105.

13. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods 2000;243:77-97.

14. Wood JC. Establishing and maintaining system linearity. Curr Protoc Cytom 2009;Chapter 1:Unit 1.4.

15. Ormerod MG. Flow Cytometry. Virtual School of Biomedical Sciences, University of Ulster.

16. Stewart CC, Stewart SJ. Four Color Compensation. Cytometry 1999;38:161-175.

17. Wood B. 9-color and 10-color flow cytometry in the clinical laboratory. Arch Pathol Lab Med 2006;130:680-690.

18. American cancer society. Global cancer facts and figures, 2nd Edition, 2008. Alanta, GA. American cancer society 2008.

19. Cancer Registry of Norway. Cancer in Norway 2008. Oslo: Cancer Registry of Norway 2009.

20. Kurman RJ, Visvanathan K, Roden R, et al. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. Am J Obstet Gynecol 2008;198:351-356.

21. Berek JS, Bast RC Jr. Ovarian cancer. In: Kufe DW, BastRC Jr. Hait WM, et al.(eds). Cancer medicine, 7th ed. Hamilton: BC Decker Inc; 2006. pp. 1543-1568.

22. Kurman RJ, Shih IeM. The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol 2010;34:433-443.

23. Permuth-Wey J, Sellers TA. Epidemiology of ovarian cancer. Methods Mol Biol 2009;472:413-437.

24. McLemore MR, Miaskowski C, Aouizerat BE, et al. Epidemiological and genetic factors associated with ovarian cancer. Cancer Nurs 2009;32:281-288.

25. Bast RC Jr, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer 2009;9:415-428.

26. Permuth-Wey J, Chen YA, Tsai YY, et al. Inherited Variants in Mitochondrial Biogenesis Genes May Influence Epithelial Ovarian Cancer Risk. Cancer Epidemiol Biomarkers Prev 2011;20:1131-1145.

27. Hennessy BT, Coleman RL, Markman M. Ovarian cancer. Lancet 2009;374:1371-1382.

28. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519-2529.

29. Crum CP. Intercepting pelvic cancer in the distal fallopian tube: theories and realities. Mol Oncol 2009;3:165-170.

30. Köbel M, Kalloger SE, Boyd N, et al. Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med. 2008;5:e232.

31. Kurman RJ, Shih IeM. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm. Hum Pathol. 2011;42:918-931.

32. Mehra K, Mehrad M, Ning G, et al. STICS, SCOUTs and p53 signatures; a new language for pelvic serous carcinogenesis. Front Biosci (Elite Ed). 2011;3:625-34.

33. Paulsen T, Kaern J, Kjaerheim K, et al. Symptoms and referral of women with epithelial ovarian tumors. Int J Gynaecol Obstet 2005;88:31-37.

34. Zaloudek C. Ovarian neoplasms. In gompel C, Silverberg SG (eds). Pathology in Gynecology and Obstetrics. Philadelphia: Lippincott;1994. pp. 330-402.

35. Tavasssoli FA, Deville P (eds). World Health Organization classification of tumors. pathology and genetics of tumors of the breast and female genital organs. Lyon: IARC Press; 2003. pp.113-145.

36. D'Angelo E, Prat J. Classification of ovarian carcinomas based on pathology and molecular genetics. Clin Transl Oncol 2010;12:783-787.

37. Heintz AP**,** Odicino F, Maisonneuve P, et al. Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. Int J Gynaecol Obstet 2006;95 Suppl 1:S161-192.

38. Ramirez I, Chon HS, Apte SM. The role of surgery in the management of epithelial ovarian cancer. Cancer Control 2011;18:22-30.

39. Ozols RF, Bundy BN, Greer BE, et al. Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. J Clin Oncol 2003;21:3194-3200.

40. du Bois A, Lück HJ, Meier W, et al. A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. J Natl Cancer Inst 2003;95:1320-1329.

41. Bookman MA. Standard treatment in advanced ovarian cancer in 2005: the state of the art. Int J Gynecol Cancer 2005;15 Suppl 3:212-220.

42. Bookman MA, Brady MF, McGuire WP, et al. Evaluation of new platinum-based treatment regimens in advanced-stage ovarian cancer: a Phase III Trial of the Gynecologic Cancer Intergroup. J Clin Oncol 2009;27:1419-1425.

43. Hoskins P, Vergote I, Cervantes A, et al. Advanced ovarian cancer: phase III randomized study of sequential cisplatin-topotecan and carboplatin-paclitaxel vs carboplatin-paclitaxel. Natl Cancer Inst 2010;102:1547-1556.

44. du Bois A, Herrstedt J, Hardy-Bessard AC, et al. Phase III trial of carboplatin plus paclitaxel with or without gemcitabine in first-line treatment of epithelial ovarian cancer. J Clin Oncol 2010;28:4162-4169.

45. Katsumata N, Yasuda M, Takahashi F, et al. Dose-dense paclitaxel once a week in combination with carboplatin every 3 weeks for advanced ovarian cancer: a phase 3, open-label, randomised controlled trial. Lancet 2009;374:1331-1338.

46. Armstrong DK, Bundy B, Wenzel L, et al. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. N Engl J Med 2006;354:34-43.

47. Rao G, Crispens M, Rothenberg ML. Intraperitoneal chemotherapy for ovarian cancer: overview and perspective. J Clin Oncol 2007;25:2867-2872.

48. Gardner GJ**,** Jewell EL. Current and future directions of clinical trials for ovarian cancer. Cancer Control 2011;18:44-51.

49. Alberts DS, Liu PY, Hannigan EV, et al. Intraperitoneal cisplatin plus intravenous cyclophosphamide versus intravenous cisplatin plus intravenous cyclophosphamide for stage III ovarian cancer. N Engl J Med 1996;335:1950-1955.

50. Markman M, Bundy BN, Alberts DS, et al. Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup study of the Gynecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. J Clin Oncol 2001;19:1001-1007.

51. Rothenberg ML, Liu PY, Braly PS, et al. Combined intraperitoneal and intravenous chemotherapy for women with optimally debulked ovarian cancer: results from an intergroup phase II trial. J Clin Oncol 2003;21:1313-1319.

52. Swart AM, Burdett S, Ledermann J, et al. Why i.p. therapy cannot yet be considered as a standard of care for the first-line treatment of ovarian cancer: a systematic review. Ann Oncol 2008;19:688-695.

53. Jaaback K, Johnson N. Intraperitoneal chemotherapy for the initial management of primary epithelial ovarian cancer. Cochrane Database Syst Rev 2006;25:CD005340.

54. Markman M. An update on the use of intraperitoneal chemotherapy in the management of ovarian cancer. Cancer J 2009;15:105-109.

55. Friedlander M. Optimally debulked stage III ovarian cancer: intraperitoneal or intravenous chemotherapy? Int J Gynecol Cancer 2010;20(11 Suppl 2):S20-23.

56. Weinberg LE, Rodriguez G, Hurteau JA. The role of neoadjuvant chemotherapy in treating advanced epithelial ovarian cancer. J Surg Oncol 2010;101:334-343.

57. Vergote I, Tropé CG, Amant F, et al. Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. N Engl J Med 2010;363:943-953.

58. du Bois A, Marth C, Pfisterer J, et al. Neoadjuvant chemotherapy cannot be regarded as adequate routine therapy strategy of advanced ovarian cancer. Int J Gynecol Cancer 2012;22:182-185.

59. Mantia-Smaldone GM, Edwards RP, Vlad AM. Targeted treatment of recurrent platinum-resistant ovarian cancer: current and emerging therapies. Cancer Manag Res 2011;3:25-38.

60. Jelovac D**,** Armstrong DK. Recent progress in the diagnosis and treatment of ovarian cancer. CA Cancer J Clin 2011;61:183-203.

61. Kalachand R, Hennessy BT, Markman M. Molecular targeted therapy in ovarian cancer: what is on the horizon? Drugs 2011;71:947-967.

62. Eskander RN, Randall LM. Bevacizumab in the treatment of ovarian cancer. Biologics 2011;5:1-5.

63. Nguyen HN, Averette HE, Hoskins W, et al. National survey of ovarian carcinoma. VI. Critical assessment of current International Federation of Gynecology and Obstetrics staging system. Cancer 1993;72:3007-3011.

64. Tropé C**,** Kaern J. Adjuvant chemotherapy for early-stage ovarian cancer: review of the literature. J Clin Oncol 2007;25:2909-2920.

65. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. Semin Surg Oncol 1994;10:31-46.

66. Sorbe B, Frankendal B, Veress B. Importance of histologic grading in the prognosis of epithelial ovarian carcinoma. Obstet Gynecol 1982;59:576-582.

67. Makar AP, Baekelandt M, Tropé CG, et al. The prognostic significance of residual disease, FIGO substage, tumor histology, and grade in patients with FIGO stage III ovarian cancer. Gynecol Oncol 1995;56:175-180.

68. Gadducci A, Cosio S, Tana R, et al. Serum and tissue biomarkers as predictive and prognostic variables in epithelial ovarian cancer. Crit Rev Oncol Hematol 2009;69:12-27.

69. Na YJ, Farley J, Zeh A, et al. Ovarian cancer: markers of response. Int J Gynecol Cancer 2009;19 Suppl 2:S21-29.

70. Maldonado L, Hoque MO. Epigenomics and ovarian carcinoma. Biomark Med. 2010;4:543-570.

71. Canevari S, Gariboldi M, Reid JF, et al. Molecular predictors of response and outcome in ovarian cancer. Crit Rev Oncol Hematol 2006;60:19-37.

72. Davidson B, Reich R, Trope CG, et al. New determinates of disease progression and outcome in metastatic ovarian carcinoma. Histol Histopathol 2010;25:1591-1609.

73. Kimmig R, Wimberger P, Hillemanns P, et al. Multivariate analysis of the prognostic significance of DNA-ploidy and S-phase fraction in ovarian cancer determined by flow cytometry following detection of cytokeratin-labeled tumor cells. Gynecol Oncol 2002;84:21-31.

74. Tropé C, Kaern J, Hogberg T, et al. Randomized study on adjuvant chemotherapy in stage I high-risk ovarian cancer with evaluation of DNA-ploidy as prognostic instrument. Ann Oncol 2000;11:281-288.

75. Silvestrini R, Daidone MG, Veneroni S, et al. The clinical predictivity of biomarkers of stage III-IV epithelial ovarian cancer in a prospective randomized treatment protocol. Cancer 1998;82:159-167.

76. Serrano-Olvera A, Dueñas-González A, Gallardo-Rincón D, et al. Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer. Cancer Treat Rev 2006;32:180-190.

77. Darcy KM, Brady WE, McBroom JW, et al. Associations between p53 overexpression and multiple measures of clinical outcome in high-risk, early stage or suboptimally-resected, advanced stage epithelial ovarian cancers A Gynecologic Oncology Group study. Gynecol Oncol 2008;111:487-495.

78. Steffensen KD, Waldstrøm M, Brandslund I, et al. Prognostic Impact of Prechemotherapy Serum Levels of HER2, CA125, and HE4 in Ovarian Cancer Patients. Int J Gynecol Cancer 2011;21:1040-1047.

79. Kong SY, Han MH, Yoo HJ, et al. Serum HE4 Level is an Independent Prognostic Factor in Epithelial Ovarian Cancer. Ann Surg Oncol 2011 Jul 21. [Epub ahead of print].

80. Davidson B, Goldberg I, Gotlieb WH, et al. Coordinated expression of integrin subunits, matrix metalloproteinases (MMP), angiogenic genes and Ets transcription factors in advanced-stage ovarian carcinoma: a possible activation pathway? Cancer Metastasis Rev 2003;22:103-115.

81. Shih IeM, Davidson B. Pathogenesis of ovarian cancer: clues from selected overexpressed genes. Future Oncol 2009;5:1641-1657.

82. Naylor B. Pleural, Peritoneal and Pericardial Fluids, 541-614. In: Bibbo M. Comprehensive Cytopathology. W. B. Saunders Company 1991.

83. Bedrossian CWM. Malignant effusions: A multimodal approach to cytologic diagnosis. New-York: Igaku-Shoin; 1994.

84. Davidson B, Firat P, Michael CW (eds). Serous effusions. Ethiology, diagnosis, prognosis and therapy. Springer; 2012.

85. Saif MW, Siddiqui IA, Sohail MA. Management of ascites due to gastrointestinal malignancy. Ann Saudi Med 2009;29:369-377.

86. Bedrossian CWM. Diagnostic problems in serous effusions. Diagn Cytopathol 1998;19:131-137.

87. Chung M**,** Kozuch P. Treatment of malignant ascites. Curr Treat Options Oncol 2008;9:215-233.

88. Becker G, Galandi D, Blum HE. Malignant ascites: systematic review and guideline for treatment. Eur J Cancer 2006;42:589-597.

89. Porcel JM**,** Vives M. Etiology and pleural fluid characteristics of large and massive effusions. Chest 2003;124:978-983.

90. Neragi-Miandoab S. Malignant pleural effusion, current and evolving approaches for its diagnosis and management. Lung Cancer 2006;54:1-9.

91. Kaifi JT, Toth JW, Gusani NJ, et al. Multidisciplinary management of malignant pleural effusion. J Surg Oncol 2011 Sep 29. doi: 10.1002/jso.22100. [Epub ahead of print].

92. Medford AR, Maskell N. Pleural effusion. Postgrad Med J 2005;81:702-710.

93. Jiménez D, Díaz G, Gil D, et al. Etiology and prognostic significance of massive pleural effusions. Respir Med 2005;99:1183-1187.

94. Bielsa S, Salud A, Martínez M, et al. Prognostic significance of pleural fluid data in patients with malignant effusion. Eur J Intern Med 2008;19:334-339.

95. Sriram KB, Relan V, Clarke BE, et al. Diagnostic molecular biomarkers for malignant pleural effusions. Future Oncol 2011;7:737-752.

96. Zahid I, Routledge T, Billè A, et al. What is the best treatment for malignant pleural effusions? Interact Cardiovasc Thorac Surg 2011;12:818-823.

97. Friedberg JS, Cengel KA. Pleural malignancies. Semin Radiat Oncol 2010;20:208-214.

98. Kassis J, Klominek J, Kohn EC. Tumor microenvironment: what can effusions teach us? Diagn Cytopathol 2005;33:316-319.

99. Dvorak HF, Weaver VM, Tlsty TD, et al. Tumor microenvironment and progression. Surg Oncol 2011;103:468-474.

100. Lorusso G, Rüegg C. The tumor microenvironment and its contribution to tumor evolution toward metastasis. Histochem Cell Biol 2008;130:1091-1103.

101. Bremnes RM, Dønnem T, Al-Saad S, et al. The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. J Thorac Oncol 2011;6:209-217.

102. Husain AN, Colby TV, Ordóñez NG, et al. Guidelines for pathologic diagnosis of malignant mesothelioma: a consensus statement from the International Mesothelioma Interest Group. Arch Pathol Lab Med 2009;133:1317-1331.

103. Risberg B, Davidson B, Nielsen S, et al. Detection of monocyte/macrophage cell populations in effusions- A comparative study using flow cytometric immunophenotyping and immunocytochemistry. Diagn Cytopathol 2001;25:214-219.

104. Fetsch PA, Abati A. Immunocytochemistry in effusion cytology. A contemporary review. Cancer 2001;93:293-308.

105. Pereira TC, Saad RS, Liu Y, et al. The diagnosis of malignancy in effusion cytology: a pattern recognition approach. Adv Anat Pathol 2006;13:174-184.

106. Davidson B. The diagnostic and molecular characteristics of malignant mesothelioma and ovarian/peritoneal serous carcinoma. Cytopathology 2011;22:5-21.

107. Ordóñez NG. What are the current best immunohistochemical markers for the diagnosis of epithelioid mesothelioma? A review and update. Hum Pathol 2007;38:1-16.

108. Sandeck HP, Røe OD, Kjærheim K, et al. Re-evaluation of histological diagnoses of malignant mesothelioma by immunohistochemistry. Diagn Pathol 2010;5:47.

109. van der Bij S, Schaake E, Koffijberg H, et al. Markers for the non-invasive diagnosis of mesothelioma: a systematic review. Br J Cancer 2011;104:1325-1333.

110. Mohanty SK, Dey P. Serous effusions: diagnosis of malignancy beyond cytomorphology. An analytic review. Postgrad Med J 2003;79:569-574.

111. Westfall DE, Fan X, Marchevsky AM. Evidence-based guidelines to optimize the selection of antibody panels in cytopathology: pleural effusions with malignant epithelioid cells. Diagn Cytopathol 2010;38:9-14.

112. Yaziji H, Battifora H, Barry TS, et al. Evaluation of 12 antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma: identification of a three-antibody immunohistochemical panel with maximal sensitivity and specificity. Mod Pathol 2006;19:514-523.

113. King JE, Thatcher N, Pickering CA, et al. Sensitivity and specificity of immunohistochemical markers used in the diagnosis of epithelioid mesothelioma: a detailed systematic analysis using published data. Histopathology 2006;48:223-232.

114. Ko EC, Jhala NC, Shultz JJ, et al. Use of a panel of markers in the differential diagnosis of adenocarcinoma and reactive mesothelial cells in fluid cytology. Am J Clin Pathol 2001;116:709-715.

115. Comin CE, Novelli L, Boddi V, et al. Calretinin, thrombomodulin, CEA, and CD15: a useful combination of immunohistochemical markers for differentiating pleural epithelial mesothelioma from peripheral pulmonary adenocarcinoma. Hum Pathol 2001;32:529-536.

116. Davidson B, Risberg B, Kristensen G, et al. Detection of cancer cells in effusions from patients diagnosed with gynaecological malignancies. Evaluation of five epithelial markers. Virchows Arch 1999;435:43-49.

117. Ordóñez NG. Value of immunohistochemistry in distinguishing peritoneal mesothelioma from serous carcinoma of the ovary and peritoneum: a review and update. Adv Anat Pathol 2006;13:16-25.

118. Murugan P, Siddaraju N, Habeebullah S, et al. Immunohistochemical distinction between mesothelial and adenocarcinoma cells in serous effusions: a combination panel-based approach with a brief review of the literature. Indian J Pathol Microbiol 2009;52:175-181.

119. Takeshima Y, Inai K, Amatya VJ, et al. Accuracy of pathological diagnosis of mesothelioma cases in Japan: clinicopathological analysis of 382 cases. Lung Cancer 2009;66:191-197.

120. Chen LM, Lazcano O, Katzmann JA, et al. The role of conventional cytology, immunocytochemistry, and flow cytometric DNA ploidy in evaluation of body cavity fluids: a prospective study of 52 patients. Am J Clin Pathol 1998;109:712-721.

121. Schneller J, Eppich E, Greenebaum E, et al. Flow cytometry and Feulgen cytophotometry in evaluation of effusions. Cancer 1987;59:1307-1313.

122. Unger KM, Raber M, Bedrossian CW, et al. Analysis of pleural effusions using automated flow cytometry. Cancer 1983;52:873-877.

123. Krishan A, Ganjei-Azar P, Jorda M, et al. Detection of tumor cells in body cavity fluids by flow cytometric and immuncytochemical analysis. Diagn Cytopathol 2006;34:528-541.

124. Sayed DM, el-Attar MM, Hussein AA. Evaluation of flow cytometric immunophenotyping and DNA analysis for detection of malignant cells in serosal cavity fluids. Diagn Cytopathol 2009;37:498-504.

125. Krishan A, Ganjei-Azar P, Hamelik R, et al. Flow immunocytochemistry of marker expression in cells from body cavity fluids. Cytometry A 2010;77:132-143.

126. Ceyhan BB, Demiralp E, Celikel T. Analysis of pleural effusions using flow cytometry. Respiration 1996;63:17-24.

127. Sikora J, Dworacki G, Trybus M, et al. Correlation between DNA content, expression of Ki-67 antigen of tumor cells and immunophenotype of lymphocytes from malignant pleural effusions. Tumour Biol 1998;19:196-204.

128. Vermeulen K, Van bockstaele DR, Berneman ZN**.** Apoptosis: mechanisms and relevance in cancer. Ann Hematol 2005;84:627-639.

129. Jin Z, El-Deiry WS. Overview of cell death signaling pathways. Cancer Biol Ther 2005;4:139-163.

130. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005;258:479-517.

131. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456-1462.

132. Viktorsson K, Lewensohn R, Zhivotovsky B. Apoptotic pathways and therapy resistance in human malignancies. Avd Cancer Res 2005;94:143-196.

133. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: A Link between Cancer Genetics and Chemotherapy. Cell 2002;108:153-164.

134. Lavrik IN. Systems biology of apoptosis signaling networks. Curr Opin Biotechnol 2010;21:551-555.

135. Chowdhury I, Tharakan B, Bhat GK. Caspases - an update. Comp Biochem Physiol B Biochem Mol Biol 2008;151:10-27.

136. Fulda S, Debatin K-M. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25:4798-4811.

137. Wang X. The expanding role of mitochondria in apoptosis. Genes & development 2001;15:2922-2933.

138. Vaux DL. Apoptogenic factors released from mitochondria. Biochim Biophys Acta 2011;1813:546-550.

139. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: The calsium-apoptosis link. Nat Rev Mol Cell Biol 2003;4:552-565.

140. Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. Oncogene 2004;23:2797-2808.

141. Guicciardi ME, Leist M, Gores GJ. Lysosomes in cell death. Oncogene 2004;23:2881-2890.

142. Ghobrial IM, Witzig TE, Adjei AA. Targeting Apoptosis Pathways in Cancer Therapy. Ca Cancer J Clin 2005;55:178-194.

143. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 2011;21:92-101.

144. Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 2011;351:41-58.

145. Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. Biol Chem 2009;284:21777-21781.

146. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000;21:485-495.

147. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277-288.

148. Krishna S, Low IC, Pervaiz S. Regulation of mitochondrial metabolism: yet another facet in the biology of the oncoprotein Bcl-2. Biochem J 2011;435:545-551.

149. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 2010;29:625-634.

150. Greijer AE**,** van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol 2004;57:1009-1014.

151. Jiang J, Tang YL, Liang XH. EMT: a new vision of hypoxia promoting cancer progression. Cancer Biol Ther 2011;11:714-723.

152. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379:88-91.

153. Seeber LM, Horrée N, Vooijs MA, et al. The role of hypoxia inducible factor-1alpha in gynecological cancer. Crit Rev Oncol Hematol 2011;78:173-184.

154. Hajra KM, Tan L, Liu JR. Defective apoptosis underlies chemoresistance in ovarian cancer. Adv Exp Med Biol 2008;622:197-208.

155. Fraser M, Leung B, Jahani-Asl A, et al. Chemoresistance in human ovarian cancer: the role of apoptotic regulators. Reprod Biol Endocrinol. 2003;1:66.

156. Kleinberg L, Davidson B. Cell survival and apoptosis-related molecules in cancer cells in effusions: a comprehensive review. Diagn Cytopathol 2009;37:613-624.

157. Bovicelli A, D'Andrilli G, Giordano A. New players in ovarian cancer. J Cell Physiol 2011;226:2500-2504.

158. Lavrik I, Golks A, Krammer PH. Death receptor signaling. J Cell Sci 2005;118:265-267.

159. Mellier G, Huang S, Shenoy K, et al. TRAILing death in cancer. Mol Aspects Med 2010;31:93-112.

160. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26-35.

161. Mahalingam D, Szegezdi E, Keane M, et al. TRAIL receptor signalling and modulation: Are we on the right TRAIL? Cancer Treat Rev 2009;35:280-288.

162. Garofalo M, Condorelli GL, Croce CM, et al. MicroRNAs as regulators of death receptors signalling. Cell Death Differ 2010;17:200-208.

163. Jackson-Bernitsas DG, Ichikawa H, Takada Y, et al. Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-kappaB activation and proliferation in human head and neck squamous cell carcinoma. Oncogene 2007;26:1385-1397.

164. Waetzig V, Loose K, Haeusgen W, et al. c-Jun N-terminal kinases mediate Fas-induced neurite regeneration in PC12 cells. Biochem Pharmacol 2008;76:1476-1484.

165. Reinehr R, Sommerfeld A, Häussinger D. CD95 ligand is a proliferative and antiapoptotic signal in quiescent hepatic stellate cells. Gastroenterology 2008;134:1494-2506.

166. Secchiero P, Melloni E, Heikinheimo M, et al. TRAIL regulates normal erythroid maturation through an ERK-dependent pathway. Blood 2004;103:517-522.

167. Choi K, Ni L, Jonakait GM. Fas ligation and tumor necrosis factor α activation of murine astrocytes promote heat shock factor-1 activation and heat shock protein expression leading to chemokine induction and cell survival. J Neurochem 2011;116:438-448.

168. Farley SM, Purdy DE, Ryabinina OP, et al. Fas ligand-induced proinflammatory transcriptional responses in reconstructed human epidermis. Recruitment of the epidermal growth factor receptor and activation of MAP kinases. J Biol Chem 2008;283:919-928.

169. Cho YS, Challa S, Clancy L, et al. Lipopolysaccharide-induced expression of TRAIL promotes dendritic cell differentiation. Immunology 2010;130:504-515.

170. Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J 2009;23:1625-1637.

171. Chen L, Park SM, Tumanov AV, et al. CD95 promotes tumour growth. Nature 2010;465:492-496.

172. Lalaoui N, Morlé A, Mérino D, et al. TRAIL-R4 promotes tumor growth and resistance to apoptosis in cervical carcinoma HeLa cells through AKT. PLoS One 2011;6:e19679.

173. Mezzanzanica D, Canevari S, Cecco LD, et al. miRNA control of apoptotic programs: focus on ovarian cancer. Expert Rev Mol Diagn 2011;11:277-286.

174. Moxley KM, Chengedza S, Benbrook DM. Induction of death receptor ligand-mediated apoptosis in epithelial ovarian carcinoma: The search for sensitizing agents. Gynecol Oncol 2009;115:438-442.

175. Duiker EW, Meijer A, van der Bilt AR, et al. Drug-induced caspase 8 upregulation sensitises cisplatin-resistant ovarian carcinoma cells to rhTRAIL-induced apoptosis. Br J Cancer 2011;104:1278-1287.

176. Bräutigam K, Biernath-Wüpping J, Bauerschlag DO, et al. Combined treatment with TRAIL and PPARγ ligands overcomes chemoresistance of ovarian cancer cell lines. J Cancer Res Clin Oncol 2011;137:875-886.

177. Li LC, Jayaram S, Ganesh L, et al. Knockdown of MADD and c-FLIP overcomes resistance to TRAIL-induced apoptosis in ovarian cancer cells. Am J Obstet Gynecol 2011;205:362.e12-25.

178. Lane D, Cartier A, L'Espérance S, et al. Differential induction of apoptosis by tumor necrosis factor-related apoptosis-inducing ligand in human ovarian carcinoma cells. Gynecol Oncol 2004;93:594-604.

179. Syed V, Mukherjee K, Godoy-Tundidor S, et al. Progesterone induces apoptosis in TRAIL-resistant ovarian cancer cells by circumventing c-FLIPL overexpression. J Cell Biochem 2007;102:442-452.

180. Horak P, Pils D, Kaider A, et al. Perturbation of the tumor necrosis factor--related apoptosis-inducing ligand cascade in ovarian cancer: overexpression of FLIPL and deregulation of the functional receptors DR4 and DR5. Clin Cancer Res 2005;11:8585-8591.

181. Tomek S, Horak P, Pribill I, et al. Resistance to TRAIL-induced apoptosis in ovarian cancer cell lines is overcome by co-treatment with cytotoxic drugs. Gynecol Oncol 2004;94:107-114.

182. Lane D, Côté M, Grondin R, et al. Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3. Mol Cancer Ther 2006;5:509-521.

183. Lane D, Robert V, Grondin R, et al. Malignant ascites protect against TRAIL-induced apoptosis by activating the PI3K/Akt pathway in human ovarian carcinoma cells. Int J Cancer 2007;121:1227-1237.

184. Abdollahi T, Robertson NM, Abdollahi A, et al. Inhibition of TRAIL-induced apoptosis by IL-8 is mediated by the p38-MAPK pathway in OVCAR3 cells. Apoptosis 2005;10:1383-1393.

185. Mezzanzanica D, Balladore E, Turatti F, et al. CD95-mediated apoptosis is impaired at receptor level by cellular FLICE-inhibitory protein (long form) in wild-type p53 human ovarian carcinoma. Clin Cancer Res 2004;10:5202-5214.

186. Baldwin RL, Tran H, Karlan BY. Primary ovarian cancer cultures are resistant to Fas-mediated apoptosis. Gynecol Oncol 1999;74:265-271.

187. Liu P, Mao H, Hou P. Synergistic antitumor effect of tumor necrosis factor-related apoptosis-inducing ligand combined with cisplatin in ovarian carcinoma cell lines in vitro and in vivo. Int J Gynecol Cancer 2006;16:538-548.

188. Vignati S, Codegoni A, Polato F, et al. Trail activity in human ovarian cancer cells: potentiation of the action of cytotoxic drugs. Eur J Cancer 2002;38:177-183.

189. Cuello M, Ettenberg SA, Nau MM, et al. Synergistic induction of apoptosis by the combination of trail and chemotherapy in chemoresistant ovarian cancer cells. Gynecol Oncol 2001;81:380-390.

190. Siervo-Sassi RR, Marrangoni AM, Feng X, et al. Physiological and molecular effects of Apo2L/TRAIL and cisplatin in ovarian carcinoma cell lines. Cancer Lett 2003;190:61-72.

191. Duiker EW, van der Zee AG, de Graeff P, et al. The extrinsic apoptosis pathway and its prognostic impact in ovarian cancer. Gynecol Oncol 2010;116:549-555.

192. Ouellet V, Le Page C, Madore J, et al. An apoptotic molecular network identified by microarray: on the TRAIL to new insights in epithelial ovarian cancer. Cancer 2007;110:297-308.

193. Lancaster JM, Sayer R, Blanchette C, et al. High expression of tumor necrosis factor-related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. Clin Cancer Res 2003;9:762-766.

194. Horak P, Pils D, Haller G, et al. Contribution of epigenetic silencing of tumor necrosis factor-related apoptosis inducing ligand receptor 1 (DR4) to TRAIL resistance and ovarian cancer. Mol Cancer Res 2005;3:335-343.

195. van Haaften-Day C, Russell P, Davies S, et al. Expression of Fas and FasL in human serous ovarian epithelial tumors. Hum Pathol 2003;34:74-79.

196. Munakata S, Enomoto T, Tsujimoto M, et al. Expressions of Fas ligand and other apoptosis-related genes and their prognostic significance in epithelial ovarian neoplasms. Br J Cancer 2000;82:1446-1452.

197. Reed J, Hakam A, Nicosia SV, et al. Significance of Fas receptor protein expression in epithelial ovarian cancer. Hum Pathol 2005;36:971-976.

198. Konno R, Takano T, Sato S, et al. Serum soluble fas level as a prognostic factor in patients with gynecological malignancies. Clin Cancer Res 2000;6:3576-3580.

199. Dobrzycka B, Terlikowski SJ, Garbowicz M, et al. Tumor necrosis factor-alpha and its receptors in epithelial ovarian cancer. Folia Histochem Cytobiol 2009;47:609-613.

200. Naylor MS, Stamp GW, Foulkes WD, et al. Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. J Clin Invest 1993;91:2194-2206.

201. Szlosarek PW, Grimshaw MJ, Kulbe H, et al. Expression and regulation of tumor necrosis factor alpha in normal and malignant ovarian epithelium. Mol Cancer Ther 2006;5:382-390.

202. Lane D, Goncharenko-Khaider N, Rancourt C, et al. Ovarian cancer ascites protects from TRAIL-induced cell death through alphavbeta5 integrin-mediated focal adhesion kinase and Akt activation. Oncogene 2010;29:3519-3531.

203. Kulbe H, Chakravarty P, Leinster DA, et al. A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment. Cancer Res. 2012;72:66-75.

204. Wu S, Boyer CM, Whitaker RS, et al. Tumor necrosis factor alpha as an autocrine and paracrine growth factor for ovarian cancer: monokine induction of tumor cell proliferation and tumor necrosis factor alpha expression. Cancer Res 1993;53:1939-1944.

205. Yuan J, Shaham S, Ledoux S, et al. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 1993;75:641-652.

206. Miura M, Zhu H, Rotello R, et al. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell 1993;75:653-660.

207. Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci 1997;22:299-306.

208. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312-1316.

209. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene 2003;22:8543-8567.

210. Crawford ED, Wells JA. Caspase substrates and cellular remodelling. Annu Rev Biochem 2011;80:1055-1087.

211. Lüthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. Cell Death Differ 2007;14:641-650.

212. Lamkanfi M, Festjens N, Declercq W,et al. Caspases in cell survival, proliferation and differentiation. Cell Death Differ 2007;14:44-55.

213. Maelfait J, Beyaert R. Non-apoptotic functions of caspase-8. Biochem Pharmacol 2008;76:1365-1373.

214. Schwerk C, Schulze-Osthoff K. Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol 2003;66:1453-1458.

215. Wagner DC, Riegelsberger UM, Michalk S, et al. Cleaved caspase-3 expression after experimental stroke exhibits different phenotypes and is predominantly non-apoptotic. Brain Res 2011;1381:237-242.

216. Irmler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. Nature1997;388:190-195.

217. Scaffidi C, Schmitz I, Krammer PH, et al. The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem 1999;274:1541-1548.

218. Bagnoli M, Canevari S, Mezzanzanica D. Cellular FLICE-inhibitory protein (c-FLIP) signalling: a key regulator of receptor-mediated apoptosis in physiologic context and in cancer. Int J Biochem Cell Biol 2010;42:210-213.

219. Krueger A, Schmitz I, Baumann S, et al. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Biol Chem 2001;276:20633-20640.

220. Budd RC, Yeh WC, Tschopp J. cFLIP regulation of lymphocyte activation and development. Nat Rev Immunol 2006;6:196-204.

221. Park SM, Schickel R, Peter ME. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. Curr Opin Cell Biol 2005;17:610-616.

222. Kataoka T, Budd RC, Holler N, et al. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr Biol 2000;10:640-648.

223. Chawla-Sarkar M, Bae SI, Reu FJ, et al. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ 2004;11:915-923.

224. Ullenhag GJ, Mukherjee A, Watson NF, et al. Overexpression of FLIPL is an independent marker of poor prognosis in colorectal cancer patients. Clin Cancer Res 2007;13:5070-5075.

225. Korkolopoulou P, Saetta AA, Levidou G, et al. c-FLIP expression in colorectal carcinomas: association with Fas/FasL expression and prognostic implications. Histopathology 2007;51:150-156.

226. Mori T, Doi R, Toyoda E, et al. Regulation of the resistance to TRAIL-induced apoptosis as a new strategy for pancreatic cancer. Surgery 2005;138:450-455.

227. Murtaza I, Saleem M, Adhami VM, et al. Suppression of cFLIP by lupeol, a dietary triterpene, is sufficient to overcome resistance to TRAIL-mediated apoptosis in chemoresistant human pancreatic cancer cells. Cancer Res 2009;69:1156-1165.

228. MacFarlane M, Harper N, Snowden RT, et al. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. Oncogene 2002;21:6809-6818.

229. Valente G, Manfroi F, Peracchio C, et al. cFLIP expression correlates with tumour progression and patient outcome in non-Hodgkin lymphomas of low grade of malignancy. Br J Haematol 2006;132:560-570.

230. Rogers KM, Thomas M, Galligan L, et al. Cellular FLICE-inhibitory protein regulates chemotherapy-induced apoptosis in breast cancer cells. Mol Cancer Ther 2007;6:1544-1551.

231. El-Gazzar A, Wittinger M, Perco P, et al. The role of c-FLIP(L) in ovarian cancer: chaperoning tumor cells from immunosurveillance and increasing their invasive potential. Gynecol Oncol 2010;117:451-459.

232. Clarke P, Tyler KL. Down-regulation of cFLIP following reovirus infection sensitizes human ovarian cancer cells to TRAIL-induced apoptosis. Apoptosis 2007;12:211-223.

233. Abedini MR, Qiu Q, Yan X, et al. Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells in vitro. Oncogene 2004;23:6997-7004.

234. Kamsteeg M, Rutherford T, Sapi E, et al. Phenoxodiol--an isoflavone analog--induces apoptosis in chemoresistant ovarian cancer cells. Oncogene 2003;22:2611-2620.

235. Korkolopoulou P, Goudopoulou A, Voutsinas G, et al. c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. Urology 2004;63:1198-1204.

236. Bullani RR, Huard B, Viard-Leveugle I, et al. Selective expression of FLIP in malignant melanocytic skin lesions. J Invest Dermatol 2001;117:360-364.

237. Du X, Bao G, He X, et al. Expression and biological significance of c-FLIP in human hepatocellular carcinomas. Exp Clin Cancer Res 2009;28:24.

238. McLornan DP, Barrett HL, Cummins R, et al. Prognostic significance of TRAIL signaling molecules in stage II and III colorectal cancer. Clin Cancer Res 2010;16:3442-3451.

239. Bagnoli M, Ambrogi F, Pilotti S, et al. c-FLIPL expression defines two ovarian cancer patient subsets and is a prognostic factor of adverse outcome. Endocr Relat Cancer 2009;16:443-453.

240. Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. Prog Lipid Res 2005;44:207-234.

241. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci 2005;62:971-988.

242. Yeung T, Gilbert GE, Shi J, et al. Membrane phosphatidylserine regulates surface charge and protein localization. Science 2008;319:210-213.

243. Stace CL**,** Ktistakis NT. Phosphatidic acid- and phosphatidylserine-binding proteins. Biochim Biophys Acta 2006;1761:913-926.

244. Chaurio RA, Janko C, Muñoz LE, et al. Phospholipids: key players in apoptosis and immune regulation. Molecules 2009;14:4892-4914.

245. Freikman I, Fibach E. Distribution and shedding of the membrane phosphatidylserine during maturation and aging of erythroid cells. Biochim Biophys Acta 2011;1808:2773-2780.

246. Utsugi T, Schroit AJ, Connor J, et al. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res 1991;51:3062-3066.

247. Fadok VA, Chimini G. The phagocytosis of apoptotic cells. Semin Immunol 2001;13:365-372.

248. Fadok VA, Voelker DR, Campbell PA, et al. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2207-2216.

249. Riedl S, Rinner B, Asslaber M, et al. In search of a novel target - phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy. Biochim Biophys Acta 2011;1808:2638-2645.

250. Schutters K**,** Reutelingsperger C. Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis 2010;15:1072-1082.

251. Homburg CH, de Haas M, von dem Borne AE, et al. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin-V binding sites during apoptosis in vitro. Blood 1995;85:532-540.

252. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995;182:1545-1556.

253. Rimon G, Bazenet CE, Philpott KL, et al. Increased surface phosphatidylserine is an early marker of neuronal apoptosis. J Neurosci Res 1997;48:563-570.

254. Schlegel RA, Williamson P. Phosphatidylserine, a death knell. Cell Death Differ 2001;8:551-563.

255. Mussunoor S, Murray GI. The role of annexins in tumour development and progression. J Pathol 2008;216:131-140.

256. Reutelingsperger CP, Hornstra G, Hemker HC. Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. Eur J Biochem 1985;151:625-629.

257. Tait JF, Gibson D, Fujikawa K. Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. J Biol Chem 1989;264:7944-7949.

258. Andree HA, Reutelingsperger CP, Hauptmann R, et al. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J Biol Chem 1990;265:4923-4928.

259. Swairjo MA, Concha NO, Kaetzel MA, et al. Ca(2+)-bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V. Nat Struct Biol 1995;2:968-974.

260. Boersma HH, Kietselaer BL, Stolk LM, et al. Past, present, and future of annexin A5: from protein discovery to clinical applications. J Nucl Med 2005;46:2035-2050.

261. Corsten MF, Hofstra L, Narula J, et al. Counting heads in the war against cancer: defining the role of annexin A5 imaging in cancer treatment and surveillance. Cancer Res 2006;66:1255-1260.

262. Vermes I, Haanen C, Steffens-Nakken H, et al. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin-V. J Immunol Methods 1995;184:39-51.

263. van Engeland M, Nieland LJ, Ramaekers FC, et al. Annexin-V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31:1-9.

264. Schmid I, Uittenbogaart C, Jamieson BD. Live-cell assay for detection of apoptosis by dual-laser flow cytometry using Hoechst 33342 and 7-amino-actinomycin D. Nat. Protoc 2007;2:187-190.

265. Hasper HJ, Weghorst RM, Richel DJ, et al. A new four-color flow cytometric assay to detect apoptosis in lymphocyte subsets of cultured peripheral blood cells. Cytometry 2000;40:167-171.

266. Davidson B, Nielsen S, Christensen J, et al. The role of desmin and N-cadherin in effusion cytology: a comparative study using established markers of mesothelial and epithelial cells. Am J Surg Pathol 2001;25:1405-1412.

267. Davidson B, Dong HP, Berner A, et al. Detection of malignant epithelial cells in effusions using flow cytometric immunophenotyping: an analysis of 92 cases. Am J Clin Pathol 2002;118:85-92.

268. van der Gun BT, Melchers LJ, Ruiters MH, et al. EpCAM in carcinogenesis: the good, the bad or the ugly. Carcinogenesis 2010;31:1913-1921.

269. Wlodkowic D, Telford W, Skommer J, et al. Apoptosis and beyond: cytometry in studies of programmed cell death. Methods Cell Biol 2011;103:55-98.

270. Galluzzi L, Aaronson SA, Abrams J, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 2009;16:1093-1107.

271. Huerta S, Goulet EJ, Huerta-Yepez S, et al. Screening and detection of apoptosis. J Surg Res 2007;139:143-156.

272. Krysko DV, Vanden Berghe T, D'Herde K, et al. Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods 2008;44:205-221.

273. Otsuki Y, Li Z, Shibata MA. Apoptotic detection methods--from morphology to gene. Prog Histochem Cytochem 2003;38:275-339.

274. Darzynkiewicz Z, Bedner E, Traganos F. Difficulties and pitfalls in analysis of apoptosis. Methods Cell Biol 2001;63:527-546.

275. Darzynkiewicz Z, Juan G, Li X, et al. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997;27:1-20.

276. Belloc F, Belaud-Rotureau MA, Lavignolle V, et al. Flow cytometry detection of caspase 3 activation in preapoptotic leukemic cells. Cytometry 2000;40:151-160.

277. Troiano L, Ferraresi R, Lugli E, et al. Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. Nat Protoc 2007;2:2719-2727.

278. van Engeland M, Ramaekers FC, Schutte B, et al. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996;24:131-139.

279. Pozarowski P, Huang X, Halicka DH, et al. Interactions of fluorochrome-labeled caspase inhibitors with apoptotic cells: a caution in data interpretation. Cytometry A 2003;55:50-60.

280. Nagata S. Apoptotic DNA fragmentation. Exp Cell Res 2000;256:12-18.

281. Darzynkiewicz Z, Zhao H. Detection of DNA strand breaks in apoptotic cells by flow- and image-cytometry. Methods Mol Biol 2011;682:91-101.

282. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 1993;53:1945-1951.

283. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, et al. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995;21:1465-1468.

284. Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995;7:61-64.

285. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980;284:555-556.

286. Yoshida A, Pommier Y, Ueda T. Endonuclease activation and chromosomal DNA fragmentation during apoptosis in leukemia cells. Int J Hematol 2006;84:31-37.

287. Oberhammer F, Wilson JW, Dive C, et al. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 1993;12:3679-3684.

288. Cohen GM, Sun XM, Snowden RT, et al. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J 1992;286 (Pt 2):331-334.

289. Saelens X, Festjens N, Vande Walle L, et al. Toxic proteins released from mitochondria in cell death. Oncogene 2004;23:2861-7284.

290. Macho A, Decaudin D, Castedo M, et al. Chloromethyl-X-Rosamine is an aldehyde-fixable potential-sensitive fluorochrome for the detection of early apoptosis. Cytometry 1996;25:333-340

291. Finucane DM, Waterhouse NJ, Amarante-Mendes GP, et al. Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. Exp Cell Res 1999;251:166-174.

292. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. Apoptosis 2003;8:115-128.

293. Fritschy JM. Is my antibody-staining specific? How to deal with pitfalls of immunohistochemistry. Eur J Neurosci 2008;28:2365-2370.

294. Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem 2011;59:13-32.

295. Turbat-Herrera EA**,** Knowles K. Cytology: screening or diagnostic tool? Hum Pathol 1998;29:1356-1366.

296. MacPhee DJ. Methodological considerations for improving Western blot analysis. J Pharmacol Toxicol Methods 2010;61:171-177.

297. Stelzer GT, Marti G, Hurley A, et al. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: standardization and validation of laboratory procedures. Cytometry. 1997;30:214-230.

298. Stewart CC, Behm FG, Carey JL, et al. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: selection of antibody combinations. Cytometry. 1997;30:231-235.

299. Kimmig R, Pfeiffer D, Landsmann H, et al. Quantitative determination of the epidermal growth factor receptor in cervical cancer and normal cervical epithelium by 2-color flow cytometry: evidence for down-regulation in cervical cancer. Int J Cancer. 1997;74:365-373.

300. van Dam PA, Lowe DG, Watson JV, et al. Multiparameter flow cytometric measurement of epidermal growth factor receptor and c-erbB-2 oncoprotein in cultured cells and in fresh and preserved solid tumor cells. Int J Gynecol Cancer. 1995;5:20-28.

301. Sethuraman C, Simmerson M, Vora AJ, et al. Flowcytometric immunophenotyping in the diagnosis of pediatric lymphoma: how reliable is it and how can we optimize its use? Pediatr Hematol Oncol. 2010;32:298-303

302. Gautam U, Srinivasan R, Rajwanshi A, et al. Comparative evaluation of flow-cytometric immunophenotyping and immunocytochemistry in the categorization of malignant small round cell tumors in fine-needle aspiration cytologic specimens. Cancer. 2008;114:494-503.

303. Sigstad E, Dong HP, Davidson B, et al. The role of flow cytometric immunophenotyping in improving the diagnostic accuracy in referred fine-needle aspiration specimens. Diagn Cytopathol. 2004;31:159-163.

304. Stomper PC, Budnick RM, Stewart CC. Use of specimen mammography-guided FNA (fine-needle aspirates) for flow cytometric multiple marker analysis and immunophenotyping in breast cancer. Cytometry. 2000;42:165-173.

305. Dressler LG, Visscher D. Handling, storage, and preparation of human tissues. Curr Protoc Cytom. 2001;Chapter 5:Unit 5.2.

306. van Dam PA, Watson JV, Lowe DG, et al. Flow cytometric measurement of cell components other than DNA: virtues, limitations, and applications in gynecologic oncology. Obstet Gynecol. 1992;79:616-21.

307. Dainiak MB**,** Kumar A**,** Galaev IY, et al. Methods in cell separations. Adv Biochem Eng Biotechnol. 2007;106:1-18.

308. McCoy JP Jr. Handling, storage, and preparation of human blood cells. Curr Protoc Cytom. 2001;Chapter 5:Unit 5.1.

309. Georgescu ES, Goldberg JM, du Plessis SS, et al. Present and future fertility preservation strategies for female cancer patients. Obstet Gynecol Surv. 2008;63:725-732.

310. Watt SM, Austin E, Armitage S. Cryopreservation of hematopoietic stem/progenitor cells for therapeutic use. Methods Mol Biol. 2007;368:237-259.

311. Pegg DE. The history and principles of cryopreservation. Cryobiology. 2002;44:46-53.

312. Koenigsmann MP, Koenigsmann M, Notter M, et al. Adhesion molecules on peripheral blood-derived CD34+ cells: effects of cryopreservation and short-term ex vivo incubation with serum and cytokines. Bone Marrow Transplant. 1998;22:1077-1085.

313. Cavers M, Afzali B, Macey M, et al. Differential expression of beta1 and beta2 integrins and L-selectin on CD4+ and CD8+ T lymphocytes in human blood: comparative analysis between isolated cells, whole blood samples and cryopreserved preparations. Clin Exp Immunol. 2002;127:60-65.

314. Honda S, Weigel A, Hjelmeland LM, et al. Induction of telomere shortening and replicative senescence by cryopreservation. Biochem Biophys Res Commun. 2001;282:493-498.

315. Terry C, Hughes RD, Mitry RR, et al. Cryopreservation-induced nonattachment of human hepatocytes: role of adhesion molecules. Cell Transplant. 2007;16:639-647.

316. Shield PW, Halford JA, Goulden R. Evaluation of a method for cryogenic storage of cytological specimens. Diagn Cytopathol. 1993;9:590-594.

317. Gonda K, Shigeura T, Sato T, et al. Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. Plast Reconstr Surg. 2008;121:401-410.

318. Liu G, Zhou H, Li Y, et al. Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells. Cryobiology. 2008;57:18-24.

319. Mittag A, Tárnok A. Basics of standardization and calibration in cytometry--a review. Biophotonics. 2009;2:470-481.

320. Schwartz A, Marti GE, Poon R, et al. Standardizing flow cytometry: a classification system of fluorescence standards used for flow cytometry. Cytometry. 1998;33:106-114.

321. Schwartz A, Fernández Repollet E, Vogt R, et al. Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. Cytometry. 1996;26:22-31.

322. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry. 2001;45:194-205.

323. Roederer M. Compensation in flow cytometry. In Robinson JP, Darzynkiewicz Z, Dean NP, Dressler GL, Rabinowitch SP, Stewart CC, Tanke JH, Wheeless LL, editors. Current protocols in cytometry, New York, John Wiley & Sons, inc., 2002;1.14.1-1.14.20.

324. Sugár IP, González-Lergier J, et al. Improved compensation in flow cytometry by multivariable optimization. Cytometry A. 2011;79:356-360.

325. Kraan J**,** Gratama JW**,** Keeney M, et al. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. J Biol Regul Homeost Agents. 2003;17:223-233.

326. Stewart CC**,** Stewart SJ. A software method for color compensation. Curr Protoc Cytom. 2003;Chapter 10:Unit 10.15

327. Maecker HT, Frey T, Nomura LE, et al. Selecting fluorochrome conjugates for maximum sensitivity. Cytometry A. 2004;62:169-173.

328. Chattopadhyay PK, Roederer M. Cytometry: Today's technology and tomorrow's horizons. Methods. 2012. [Epub ahead of print].

329. Stewart CC, Stewart SJ. Titering antibodies. Curr Protoc Cytom. 2001;Chapter 4:Unit 4.1.

330. Hulspas R. Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells. Curr Protoc Cytom. 2010;Chapter 6:Unit 6.29.

331. Mahnke YD**,** Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med. 2007;27:469-485.

332. Hulspas R, O'Gorman MR, Wood BL, et al. Considerations for the control of background fluorescence in clinical flow cytometry. Cytometry B Clin Cytom. 2009;76:355-364.

333. Koester SK, Bolton WE. Strategies for cell permeabilization and fixation in detecting surface and intracellular antigens. Methods Cell Biol. 2001;63:253-268.

334. Lanza F, Latorraca A, Moretti S, et al. Comparative analysis of different permeabilization methods for the flow cytometry measurement of cytoplasmic myeloperoxidase and lysozyme in normal and leukemic cells. Cytometry. 1997;30:134-144.

335. Macey MG, McCarthy DA, Milne T, et al. Comparative study of five commercial reagents for preparing normal and leukaemic lymphocytes for immunophenotypic analysis by flow cytometry. Cytometry. 1999;38:153-160.

336. Stewart JC, Villasmil ML, Frampton MW. Changes in fluorescence intensity of selected leukocyte surface markers following fixation. Cytometry A. 2007;71:379-385.

337. Lecoeur H, Ledru E, Gougeon ML. A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. J Immunol Methods. 1998;217:11-26

338. Kappelmayer J, Gratama JW, Karászi E, et al. Flow cytometric detection of intracellular myeloperoxidase, CD3 and CD79a. Interaction between monoclonal antibody clones, fluorochromes and sample preparation protocols. J Immunol Methods. 2000;242:53-65.

339. da Costa ES, Peres RT, Almeida J, et al. Harmonization of light scatter and fluorescence flow cytometry profiles obtained after staining peripheral blood leucocytes for cell surface-only versus intracellular antigens with the Fix & Perm reagent. Cytometry B Clin Cytom. 2010;78:11-20.

340. Van Ewijk W, Van Soest PL, Verkerk A, et al. Loss of antibody binding to prefixed cells: fixation parameters for immunocytochemistry. Histochem J.1984;16:179-193.

341. Ciaravino G, Bhat M, Manbeian CA, et al. Differential expression of CD40 and CD95 in ovarian carcinoma. Eur J Gynaecol Oncol. 2004;25:27-32.

342. Lane D, Matte I, Rancourt C, et al. The prosurvival activity of ascites against TRAIL is associated with a shorter disease-free interval in patients with ovarian cancer. J Ovarian Res. 2010;3:1.

343. Connor JP**,** Felder M. Ascites from epithelial ovarian cancer contain high levels of functional decoy receptor 3 (DcR3) and is associated with platinum resistance. Gynecol Oncol. 2008;111:330-335.

344. Mace TA, Yamane N, Cheng J, et al. The potential of the tumor microenvironment to influence Apo2L/TRAIL induced apoptosis. Immunol Invest. 2006;35:279-296.

345. Mayes PA, Campbell L, Ricci MS, et al. Modulation of TRAIL-induced tumor cell apoptosis in a hypoxic environment. Cancer Biol Ther. 2005;4:1068-1074.

346. Sayers TJ. Targeting the extrinsic apoptosis signaling pathway for cancer therapy. Cancer Immunol Immunother. 2011;60:1173-1180.

347. Takeda K, Stagg J, Yagita H, et al. Targeting death-inducing receptors in cancer therapy. Oncogene. 2007;26:3745-3757.

348. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer. 2002;2:420-430.

349. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol. 2004;4:333-339.

350. Roberts NJ, Zhou S, Diaz LA Jr, et al. Systemic use of tumor necrosis factor alpha as an anticancer agent. Oncotarget. 2011;2:739-751.

351. Amm HM, Oliver PG, Lee CH, et al. Combined modality therapy with TRAIL or agonistic death receptor antibodies. Cancer Biol Ther. 2011;11:431-449.

352. Khaider NG, Lane D, Matte I, et al. Targeted ovarian cancer treatment: the TRAILs of resistance. Am J Cancer Res. 2012;2:75-92.

353. Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol. 2008;26:3621-3630.

354. Holland PM. Targeting Apo2L/TRAIL receptors by soluble Apo2L/TRAIL. Cancer Lett. 2011; [Epub ahead of print]

355. Estes JM, Oliver PG, Straughn JM Jr, et al. Efficacy of anti-death receptor 5 (DR5) antibody (TRA-8) against primary human ovarian carcinoma using a novel ex vivo tissue slice model. Gynecol Oncol. 2007;105:291-298.

356. Frederick PJ, Kendrick JE, Straughn JM Jr, et al. Effect of TRA-8 anti-death receptor 5 antibody in combination with chemotherapy in an ex vivo human ovarian cancer model. Int J Gynecol Cancer. 2009;19:814-819.

357. Kleinberg L, Dong HP, Holth A, et al. Cleaved caspase-3 and nuclear factor-kappaB p65 are prognostic factors in metastatic serous ovarian carcinoma. Hum Pathol. 2009;40:795-806.

358. Davidson B, Reich R, Lazarovici P, et al. Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. Clin Cancer Res. 2003;9:2248-5229.

359. Sodek KL, Murphy KJ, Brown TJ, et al. Cell-cell and cell-matrix dynamics in intraperitoneal cancer metastasis. Cancer Metastasis Rev. 2012;31:397-414.

360. Pepper C, Thomas A, Tucker H, et al. Flow cytometric assessment of three different methods for the measurement of in vitro apoptosis. Leuk Res. 1998;22:439-444.

361. Overbeeke R, Steffens-Nakken H, Vermes I, et al. Early features of apoptosis detected by four different flow cytometry assays. Apoptosis. 1998;3:115-121.

362. Fadeel B, Gleiss B, Högstrand K, et al. Phosphatidylserine exposure during apoptosis is a cell-type-specific event and does not correlate with plasma membrane phospholipid scramblase expression. Biochem Biophys Res Commun. 1999;266:504-511.

363. Balasubramanian K, Mirnikjoo B, Schroit AJ. Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. Biol Chem. 2007;282:18357-18364.

364. Pap E. The role of microvesicles in malignancies. Adv Exp Med Biol. 2011;714:183-199.

365. Hammill AK, Uhr JW, Scheuermann RH. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. Exp Cell Res. 1999;251:16-21.

366. Yang MY, Chuang H, Chen RF, et al. Reversible phosphatidylserine expression on blood granulocytes related to membrane perturbation but not DNA strand breaks. J Leukoc Biol. 2002;71:231-237.

367. Lin SH, Vincent A, Shaw T, et al. Prevention of nitric oxide-induced neuronal injury through the modulation of independent pathways of programmed cell death. J Cereb Blood Flow Metab. 2000;20:1380-1391.

368. Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. Cancer Biol Ther. 2005;4:924-933.

369. Moustakas A, Pardali K, Gaal A, et al. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett. 2002;82:85-91.

370. Lima LG, Chammas R, Monteiro RQ, et al. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. Cancer Lett. 2009;283:168-175.

371. Kim HK, Song KS, Park YS, et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. Eur J Cancer. 2003;39:184-191.

372. Ginestra A, Miceli D, Dolo V, et al. Membrane vesicles in ovarian cancer fluids: a new potential marker. Anticancer Res. 1999;19:3439-3445.

373. Safa AR, Pollok KE. Targeting the Anti-Apoptotic Protein c-FLIP for Cancer Therapy. Cancers (Basel). 2011;3:1639-1671.

374. Wang W, Wang S, Song X, et al. The relationship between c-FLIP expression and human papillomavirus E2 gene disruption in cervical carcinogenesis. Gynecol Oncol. 2007;105:571-577.

375. Tian F, Lu JJ, Wang L, et al. Expression of c-FLIP in malignant melanoma, and its relationship with the clinicopathological features of the disease. Clin Exp Dermatol. 2012;37:259-265.

376. Valnet-Rabier MB, Challier B, Thiebault S, et al. c-Flip protein expression in Burkitt's lymphomas is associated with a poor clinical outcome. Br J Haematol. 2005;128:767-773.

377. Li X, Pan X, Zhang H, et al. Overexpression of cFLIP in head and neck squamous cell carcinoma and its clinicopathologic correlations. J Cancer Res Clin Oncol. 2008;134:609-615.

378. Zhou XD, Yu JP, Liu J, et al. Overexpression of cellular FLICE-inhibitory protein (FLIP) in gastric adenocarcinoma. Clin Sci (Lond). 2004;106:397-405.

379. Mathas S, Lietz A, Anagnostopoulos I, et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med. 2004;199:1041-1052.

380. Haag C, Stadel D, Zhou S, Bachem MG, et al. Identification of c-FLIP(L) and c-FLIP(S) as critical regulators of death receptor-induced apoptosis in pancreatic cancer cells.Gut. 2011;60:225-237.

381. Bagnoli M, Balladore E, Luison E, et al. Sensitization of p53-mutated epithelial ovarian cancer to CD95-mediated apoptosis is synergistically induced by cisplatin pretreatment. Mol Cancer Ther. 2007;6:762-772.

382. Abedini MR, Muller EJ, Brun J, et al. Cisplatin induces p53-dependent FLICE-like inhibitory protein ubiquitination in ovarian cancer cells. Cancer Res. 2008;68:4511-4517.

383. Abedini MR, Muller EJ, Bergeron R, et al. Akt promotes chemoresistance in human ovarian cancer cells by modulating cisplatin-induced, p53-dependent ubiquitination of FLICE-like inhibitory protein. Oncogene. 2010;29:11-25.

384. Takai N, Kawamata N, Gui D, et al. Human ovarian carcinoma cells: histone deacetylase inhibitors exhibit antiproliferative activity and potently induce apoptosis. Cancer. 2004;101:2760-2770.

385. Park SJ, Kim MJ, Kim HB, et al. Trichostatin A sensitizes human ovarian cancer cells to TRAIL-induced apoptosis by down-regulation of c-FLIPL via inhibition of EGFR pathway. Biochem Pharmacol. 2009;77:1328-1336.

386. Frankel A, Man S, Elliott P, et al. Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. Clin Cancer Res. 2000;6:3719-3728.

387. Zhu H, Zhang L, Dong F, et al. Bik/NBK accumulation correlates with apoptosis-induction by bortezomib (PS-341, Velcade) and other proteasome inhibitors. Oncogene. 2005;24:4993-4999.

388. Saulle E, Petronelli A, Pasquini L, et al. Proteasome inhibitors sensitize ovarian cancer cells to TRAIL induced apoptosis. Apoptosis. 2007;12:635-655.

389. Falschlehner C, Emmerich CH, Gerlach B, et al. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol. 2007;39:1462-1475.

9. ERRATA

9.1. Post-submission errata approved by the evaluation committee

9.1.1. Spelling, grammar, omissions and additions

Page 63 line 3: “detailed in **Table 2**” was changed to “detailed in **Table 3**”

Page 63 table headline: “**Table 2. Cell lines used in the thesis”** was changed to “**Table 3. Cell lines used in the thesis**”

Page 69 line 17: “studied in papers II-V” was changed to “studied in papers III and V”

Page 69 line 25: “fluorochrome-labeled triphoshodeoxynucleotides” was changed to “fluorochrome-labeled triphosphodeoxynucleotides”

Page 82 line 20: “matrix mellaproteinases at the in cultured cryopreserved hepatocytes” was changed to ” matrix mellaproteinases in the cultured cryopreserved hepatocytes”

Page 95 line 7: ”IV” was changed to ”VI”

I

II

III

IV

V

VI